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THE EFFECT OF ENVIRONMENTAL FACTORS ON
THE PHYSIOLOGY OF *AEROMONAS HYDROPHILA*
IN LAKE WATER

By

CHAE-HONG LIM

A thesis presented for the degree of
Doctor of Philosophy

Department of Biological Science,
University of Warwick

August , 1995

Declaration

I declare that all of the work contained in this thesis is my own, unless otherwise acknowledged, and has not been used in any previous application for a degree.

Chae-Hong Lim

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Abbreviations

μm	10^{-6} meter
l	Litre
μl	10^{-6} litre
nm	10^{-9} meter
%	Percentage
c.f.u.	Colony forming units
g	Gram
g	Gravitational force
g/l	Gram per litre
g-C/l	Gram carbon per litre
g-N/l	Gram nitrogen per litre
d	Day(s)
h	Hour(s)
min	Minute(s)
M	Molarity
kDa	Kilodalton
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
mA	10^{-3} amper
mg	10^{-3} gram
μg	10^{-6} gram
mg/l	10^{-3} gram per litre
mg/ml	10^{-3} gram per 10^{-3} litre
pNPP	4-nitrophenyl disodium orthophosphate
rpm	Revolutions per minute
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid

ppGpp	Guanosine-3'-diphosphate-5'-diphosphate
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEMED	N'N'N' tetramethyl ethylene diamine
Tris	Tris-(hydroxymethyl)-methylamine
TES	N-tris(hydroxymethyl)methyl-2-amino ethane sulphonic acid
V	Volt
w	Weight
v	Volume
TTA	TES-Tris-Acetic acid
EDTA	Diamino ethane tetraacetic acid
T ₉₀	The time taken for a 1 log drop in numbers from initial viable count
T ₉₉	The time taken for a 2 log drop in numbers from initial viable count
PLM	Phosphate-limited minimal medium
PRM	Phosphate-rich medium (= nutrient broth)
TE	Tris-EDTA buffer
HSD	Honestly significant difference

Summary

The survival and physiological responses of *A. hydrophila* were investigated in lake water microcosms under starvation and other stress conditions. Longer survival was shown in filtered-autoclaved water than in Whatman-filtered water than in untreated water. Longer survival also occurred at 4° than at 15° than at 25° than at 30° than at 37°C. The enhanced survival times over unamended controls suggesting that protozoa could be involved in regulating the size of the population of *A. hydrophila*. Nutrient amendments such as synthetic sewage, casein, ammonium sulphate, serine and glutamine also increased survival of *A. hydrophila* over that in unamended controls. Organic compounds released from *Flavobacterium*, *Anacystis*, and a number of algae also increased survival times of *A. hydrophila* in filtered-autoclaved lake water microcosms. Cell size was reduced under starvation conditions and the numbers of cells capable of respiration also decreased but was always greater than the viable count especially if cells were starved at 37°C. This suggests that *A. hydrophila* is capable of entering a viable but non-culturable phase under starvation conditions. The effects of starvation on two enzymes, phosphatase and exoprotease, both important in the scavenging of nutrients were examined. The activity of alkaline phosphatase and exoprotease both increased under starvation stress with the largest increases being seen at higher starvation temperatures although the viable count often decreased below the limits of detection at the same time. Nutrient amendments, such as a variety of carbon and nitrogen sources, led to an increase in activity of both enzymes and also led to induction of alkaline phosphatase activity in cells grown in high phosphate medium to repress alkaline phosphatase activity. This is an obvious indicator that derepression of alkaline phosphatase and synthesis of the enzyme could occur under these conditions in lake water microcosms. Exoprotease activity was also increased upon the addition of single nutrient source to the microcosms. Osmotic stress coupled with starvation stress also increased alkaline phosphatase activity and the addition of the osmoprotectant, betaine, also increased activity. In both cases activity increased although the viable count decreased, in some cases below the limits of detection. Exoprotease activity increased in osmotically shocked cells and increased further if betaine was added to the starvation medium. Plasmid transfer could still occur between *A. hydrophila* and *Escherichia coli* in unamended and lake water microcosms amended with carbon and some nitrogen sources. The transfer of the F group plasmid R1d₁d19 was temperature dependent with no detectable transfer occurring at temperatures below 15° C even in nutrient broth. Plasmid transfer was dependent upon the size of the donor and recipient populations with no detectable transfer occurring at low population densities but transfer at high densities shows that even under prevailing environmental conditions the transfer of F-plasmids was possible between *E. coli* and *A. hydrophila*. The changes in protein fingerprints of cell and periplasm extracts under starvation and other stresses were examined using two-dimensional gel electrophoresis. Several starvation specific proteins were identified on the gels and some proteins which were only transiently produced were noted. The N-terminal sequences of two stress proteins produced in response to starvation and ethanol and heat stress were obtained from the gels. Alkaline phosphatase one of the key proteins in the response to stress was also identified by colourimetric staining of two-dimensional gels. The survival of *A. hydrophila* under starvation and other stresses is dependent upon the sequential synthesis of many proteins, including alkaline phosphatase.

Chapter 1

General Introduction

1.1 Bacteriology

A single strain of the genus *Aeromonas* was first isolated from drinking water supplies in the city of Chemnitz by Zimmermann in 1890 and exhibited typical dotted growth on gelatin agar, being described as "*Bacillus punctatus*". After several changes of its name, the microorganism was finally classified as *Aeromonas hydrophila* in the seventh edition of Bergey's Manual (Snieszko, 1957).

Aeromonas spp. are important members of the normal aquatic microflora and are widely dispersed occurring in various habitats, such as soil, fresh water, sea water, drinking water, swimming pools, thermally altered water, sewage and faeces of animals (Hazen & Fliermans, 1979; Palhak *et al.*, 1988; Araujo *et al.*, 1989, 1990, 1991). *Aeromonas* spp. have been found to be the most prevalent bacteria in raw water (16% of the bacterial population) and the second largest bacterial population in chlorinated distribution water (LeChevallier *et al.*, 1980). *Aeromonas* are Gram-negative facultatively anaerobic rods, 0.3 - 1.0 μm in diameter and 1.0 - 4.4 μm in length. They are motile by a single polar flagellum. They are chemoorganotrophic using a variety of sugars and organic acids as carbon sources, and have been reported as growing on very low concentrations of carbon compounds in drinking water. They have a growth temperature range of 5° to 41°C with an optimum of 28°C. On nutrient agar, colonies of motile aeromonads are round, raised, with an entire edge and have a smooth surface. They are translucent and white to buff in colour (Popoff, 1984). Two well separated groups are included in the genus *Aeromonas*. The first group consists of mesophilic and motile bacteria. This group can be divided into three species; *A. hydrophila*, *A. caviae* and *A. sobria*. The psychrophilic and non-motile aeromonads are clustered in the second group named *A. salmonicida* (Popoff, 1984; Khardori & Fainstein, 1988). In addition, four novel motile species were taxonomically proposed with differentiation based on a few biochemical

characteristics. Table 1.1 lists some of the biochemical characteristics of the three type species and four proposed species of motile *Aeromonas* spp.

Table 1.1 Biochemical tests used to differentiate motile *A. hydrophila* spp.

(From Ogden *et al.*, 1994)

Test	Reaction of different motile <i>Aeromonas</i> spp.						
	<i>Aeromonas hydrophila</i>	<i>Aeromonas caviae</i>	<i>Aeromonas hydrophila</i>	<i>Aeromonas salmonicida</i>	<i>Aeromonas schubertii</i>	<i>Aeromonas trota</i>	<i>Aeromonas trota</i>
Aesculin hydrolysis	+	+	-	+	-	-	-
Growth in KCN broth	+	+	-	-	-	NT	NT
Histidine utilization	+	+	-	NT	NT	NT	NT
Arginine utilization	+	+	-	NT	NT	NT	NT
Mannitol fermentation	+	+	+	+	-	+	+
Salicin fermentation	+	+	-	+	-	-	-
Sucrose fermentation	+	+	+	+	-	-	-
VP	+	-	d	+	-	+	-
Gas from glucose	+	-	+	+	-	+	+
H ₂ S from cysteine	+	-	+	NT	NT	+	+
Lysine decarboxylase	+	-	+	+	+	+	+
Ornithine decarboxylase	-	-	-	+	-	-	-
Arginine dihydrolase	+	+	+	-	+	+	+
Ampicillin resistance	+	+	+	+	+	+	-

+, Positive

-, negative

d, 11-89% of strains are positive.

NT, Not tested

In vitro determination of the minimal inhibitory concentrations (MIC) of 32 antimicrobial agents by a microdilution method showed *Aeromonas* isolates from patients are usually susceptible to chloramphenicol, tetracycline, gentamycin and relatively resistant to penicillins, erythromycin and ampicillin (Table 1.2; Fass & Barnishan, 1981). Most *Aeromonas* spp. are resistant to several antibiotics, including ampicillin which is often used in *Aeromonas* selective media at concentrations between 10 and 30 µg/ml. Several media for the selective isolation of *Aeromonas* spp. have been reported by Arcos *et al.* (1988) and Ribas *et al.* (1991).

Table 1.2 *In vitro* susceptibilities of *A. hydrophila* to 32 antimicrobial agents

(From Fass & Barnishan, 1981)

Antimicrobial agents	Minimal inhibitory concentration ($\mu\text{g/ml}$)		
	Range	50% inhibition	90% inhibition
Penicillin G	64 - > 128	> 128	> 128
Ampicillin	16 - > 128	> 128	> 128
Carbenicillin	8 - > 128	> 128	> 128
Ticarcillin	8 - > 128	> 128	> 128
Azlocillin	8 - > 128	32	64
Mezlocillin	4 - > 128	8	8
Pipercillin	1 - > 128	4	8
Mecillinam	0.25 - 4	1	3
Cephalexin	2 - > 128	> 128	> 128
Cefazolin	4 - > 128	128	> 128
Cefamandole	0.25 - 32	2	8
Cefoxitin	0.5 - 128	8	32
Cefazafur	0.5 - > 128	64	> 128
Cefuroxime	0.25 - 8	1	4
Cefotaxime	≤ 0.06 - 4	≤ 0.06	0.25
Moxalactam	≤ 0.06	≤ 0.06	≤ 0.06
Streptomycin	2 - > 64	8	16
Kanamycin	0.5 - 8	2	8
Gentamicin	0.13 - 0.5	0.25	0.5
Tobramycin	0.13 - 2	1	2
Amikacin	0.5 - 4	2	4
Sisomicin	0.13 - 1	0.5	1
Netilmicin	0.13 - 1	0.5	1
Colistin	1 - > 64	4	> 64
Tetracycline	0.5 - 2	1	2
Minocycline	0.5 - 4	1	4
Doxycycline	0.5 - 4	1	2
Chloramphenicol	0.5 - 8	1	4
Erythromycin	8 - 64	16	32
Clindamycin	264	> 64	> 64
Vancomycin	264	> 64	> 64
TMP-SMZ*	2 - 32	4	8

TMZ - SMZ*, Total trimethoprim - sulfamethoxazole in a ratio of 1:19

1.2 Epidemiology and clinical significance

Aeromonas has been recognised as a pathogen of amphibians, reptiles, fish, snails, cows and humans. These organisms are not part of the human flora and the rare *Aeromonas* isolates from stools of healthy persons are usually transient (Khadori & Fainstein, 1988; Deodhar *et al.*, 1991). Burke *et al.* (1984a; 1984b) showed that cases of *Aeromonas*-associated diarrhoea paralleled the isolation of

Aeromonas spp. from both chlorinated and unchlorinated domestic water supplies, with both being greatest during summer. Kuijper *et al.* (1987) detected *Aeromonas* from faecal samples and isolated them on a semi-selective blood agar plate with 10 µg/ml ampicillin. Of 156 *Aeromonas* isolates, 15% were identified as *A. hydrophila*, 17% as *A. sobria* and 68% as *A. caviae*. There was a good correlation between the isolation of *A. hydrophila* and *A. sobria*, and the clinical symptoms of gastroenteritis or enterocolitis.

Aeromonas spp., especially *A. hydrophila* and *A. sobria* were implicated as causative agents of human gastroenteritis particularly in infants and in the elderly in the summer months (Gracey *et al.*, 1982). Gastrointestinal infections are usual self-limiting in normal individuals and remain localised. However dissemination is more likely to occur in immunocompromised patients and may result in intra-abdominal infection or septicemia (Khardori & Fainstein, 1988). Holmberg *et al.* (1986) isolated *A. hydrophila* in large numbers from patients who were more likely to have drunk untreated water, usually from private wells and found that patients who took antibiotics to which *Aeromonas* were susceptible experienced alleviation or resolution of their gastro-intestinal symptoms. These observations indicate that *Aeromonas* are enteropathogenic for the normal host and acquired by drinking untreated water. Skin and soft tissue infections are the second most common site for *A. hydrophila* isolation after the gastrointestinal tract. Most of these infections are directly associated with exposure to water or soil. Most non-enteric *Aeromonas* infections occur in open wounds or as the result of fractures during the recreational use of contaminated water.

Aeromonas spp. produce a variety of biologically active extracellular products such as enterotoxins, cytotoxins, hemolysins, a cytotoxic hemolysin, and aerolysin some of which are much more frequently found in isolates obtained from patients with gastrointestinal symptoms than without (Gracey *et al.*, 1982; Chopra *et al.*, 1991). It was first proposed by Burke *et al.* (1982) that enterotoxin production in *Aeromonas* spp. was associated with biotype. Burke *et al.* (1984b)

found that 70.2% of their strains isolated from water were enterotoxigenic in the suckling mouse test while earlier studies reported that all strains isolated from drinking water, river water and sewage were enterotoxigenic (Dubey and Sanyal, 1979). Hemolysin production of *Aeromonas* correlated closely with enterotoxin production (Kirov *et al.*, 1986). Results of many studies of enterotoxin production and classification by species of *Aeromonas* isolated from water are conflicting. Kaper *et al.* (1981) examined 118 isolates from the Chesapeake Bay in America and all were classified as *A. hydrophila*. A total of 71% (83 of 116) produced cytotoxin and 73% (8 of 11) gave a positive reaction for enterotoxin production in the rabbit ileal loop test. LeChevallier *et al.* (1982) investigated species isolated from chlorinated drinking water and classified all 20 isolates as *A. sobria*. 80% (16 of 20) produced cytotoxins but were negative in the rabbit ileal loop assay. Clearly there are marked regional differences in the prevalence of different species of *Aeromonas* in the proportion of enterotoxigenic strains. Water temperature, residual chlorine levels and interaction between these variables were shown to influence the growth of *Aeromonas* and probably contribute to such differences (Burke *et al.*, 1984a; Kirov *et al.*, 1986).

1.3 Survival strategies of micro-organisms in natural waters

1.3.1 The aquatic environment as a microbial habit

About 97% of Earth's water is in the oceans, 2% in glaciers and polar ice, 0.009% in lakes, 0.00009% in rivers, and the residue in ground waters. Water is the dominant environment as the oceans cover 71% of the globe's surface. Inland surface waters are separable into flowing (lotic) and still (lentic) environments. The lotic streams and rivers provide relatively uniform environments throughout their depths, whereas, in contrast, lentic lakes develop distinct water layers differing in temperature, density, chemistry and biology (Grant & Long, 1981).

Water has a very high specific thermal capacity. Consequently, large bodies of water absorb or lose a great amount of heat energy without much change in temperature and many aquatic organisms enjoy very stable climates. Because of the water temperature-density relationship, freshwater at approximately 4°C sinks, and the bottoms of large ponds, lakes and their sediments are rarely frozen (Grant & Long, 1981). Aquatic environments are subject to large spatial and temporal nutrient fluxes arising from seasonal and geographic variations in temperature, salinity, nutrient input (Rozak & Colwell, 1987). The main zones of a lake are shown in Fig 1.1 (adapted from Grant & Long, 1981). Stratification commonly results from surface heating when turbulence is low enough to prevent mixing of the water column. The base of the warmed upper layer is indicated by an abrupt drop in temperature, the thermocline. In stratified waters, the vertical movement of solutes and gases across the thermocline is very slow. As a result, organic and inorganic nutrients released by decay below the thermocline are not returned to the euphotic zone, while stratification remains. At the same time, decomposition in the deeper water can lead to oxygen depletion and the accumulation of fermentation products and of hydrogen sulphide. The summer appearance of an oxygen-limited lower zone (hypolimnion) is characteristic of nutrient-rich, relatively shallow eutrophic lakes (lakes producing 75-250 g fixed C m⁻²y⁻¹). Re-entry of nutrients into the euphotic zone occurs when the thermocline is destroyed by autumn gales, and is often marked by an algal bloom as nutrients become available (Grant & Long, 1981).

Aquatic habitats are listed in Table 1.3 (from Grant & Long, 1981). The air-water interface (neustonic habitat) is the most extreme and unusual. It is the site of a detergent-like film of hydrophobic hydrocarbons, fatty acids and esters overlying a layer of polysaccharide-protein complexes just below the interface. Liquid-solid interfaces are also regions where nutrients and carbon sources may accumulate through sorption. Microorganisms can be attracted to such enriched surfaces and be retained there by sorption (Grant & Long, 1981). Kjelleberg *et*

al.(1982) also reported that in the natural aquatic environment, microbial habitats are abundant and varied and adjacent habitats may be very different even though in direct juxtaposition, some consisting of expanses of liquid bounded by interfaces with solid gaseous, or rather liquid phases of different densities. The interfaces are sites where nutrients accumulate, allowing the microbes there to undergo active growth.

Bacteria that are able to reach a nutrient-rich interface by chemotatic response, motility, or random movement have a selective advantage in an otherwise nutrient-poor environment. In addition to those bacteria able to attach to solid surfaces at a liquid-solid interface, other bacteria can loosely associate with surfaces temporarily by reversible sorption, thereby taking advantage of the high-nutrient environment adjacent to the actual surface (Marshall, 1979). Grant and Long (1981) reported that the bacteria most commonly isolated from the aquatic environment are Gram-negative rods, and indeed 90% of all isolates fall into this category, usually including species of *Vibrio*, *Pseudomonas* and *Flavobacterium* or related organisms.

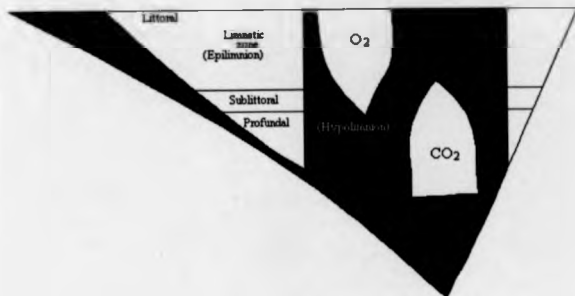


Figure 1.1 Zonation in a stratified eutrophic lake. The thermocline is positioned between the O_2 -rich epilimnion and the O_2 -depleted hypolimnion. (From Grant & Long, 1981)

Table 1.3 Microbial habits

(Grant & Long, 1981)

Habitat	Definition	Community terms	Sub-divisions
1. Neustonic	The surface film (air-water interface)	Neuston	
2. Planktonic	The water column, its free-floating inhabitants of limited motility	Plankton	Potamoplankton (= river plankton)
3. Benthic	In and on permanently submerged sediments	Benthos	(a) Psammion or Hydropsammion (interstitial water between sand grains) (b) Epipsammion (on sand grains) (c) Epieplon (on mud)
4. Epibiotic	On living and non-living surfaces large enough to support a mixed microbial community	Periphyton (all attached organisms)	(a) Epiphyton (on plants) (b) Epizoon (on animals) (c) Epilithon (on rocks and stones)
5. Enteric-faecal sestonic	Within animal guts and continuing on faeces, also on any floating particulate organic matter	Enteric faecal seston	

1.3.2 The growth of bacteria in the aquatic environment

Water bacteria play an indispensable role in nutrient cycles and chemical and ecological processes. The nutrient status of natural waters is subject to multiple limitations associated with oscillatory concentrations of carbon, phosphate, nitrogen, sulphate and trace elements which vary in conjunction with environmental factors such as temperature, redox potential and pH (Morgan & Dow, 1986). In the survey of nutrient amounts, Webb & D'elia (1980) determined, in the Chesapeake Bay, that bay water contains, per litre: 10 to 90 μg -atoms of N, including NO_3^- , NH_4^+ , urea N, and NO_2^- ; 0.6 to 1.8 mg-atoms of P; and about 15 mg of dissolved organic carbon. Yanagita (1978) *et al.* obtained data on nutrient concentrations; 2.4-3.5 mg of dissolved organic carbon, 0.5-1.0 mg

of total nitrogen and 0.05-0.1 mg of total phosphorus, per litre, in bay water, and 0.5-1.0 mg of dissolved organic carbon, 0.1-0.15 mg of total nitrogen and 0.01 mg of total phosphorus, per litre in sea water. In low nutrient environments the growth of aquatic bacteria is constrained by a combination of these parameters rather than by carbon content alone and this is reflected in the physiology of the bacteria inhabiting these systems (Morgan & Dow, 1986).

1.3.2.1 Carbon-limited growth

In the laboratory carbon-substrate limitation has been employed to mimic environmental conditions most commonly in studies of microbial response to nutrient constraint in chemoorganotrophs. A condition of carbon-substrate limitation in chemoorganotrophs is characterized by a high carbon conversion efficiency in which, in most organisms, the diversion of substrate carbon into extracellular products is minimized (Tempest & Wouters, 1981). Under such conditions organisms tend to depress the synthesis of their catabolic enzyme machinery, whereas the synthesis of anabolic functions remains adjusted to levels in keeping with the growth rate. One consequence of this behaviour is that although the rates of catabolism and anabolism may be adequately tuned to a particular condition of nutrient constraint, organisms will frequently catabolize at a high rate any excess substrate suddenly added to such a culture (Neijssel & Tempest, 1979). Heterotrophic aquatic bacteria are often adapted to low levels of nutrients. Oligotrophic growth, as defined by the ability of bacteria to grow in media containing 1 mg or less of organic carbon per litre, as well as extended longevity under nutrient starvation, has been shown to represent physiological adaptations to low nutrient environments (Jones & Rhodes-Roberts, 1981). For production of new cellular material (assimilation) and as an energy source (dissimilation) organic carbon is utilized by heterotrophic bacteria. Most organic carbon in water supplies is natural in origin and is derived from living and decaying

vegetation. These compounds may include humic and fulvic acids, polymeric carbohydrates, protein, and carboxylic acids. The total organic carbon concentration of finished drinking water in 80 locations in the U.S. ranged from 0.05 to 12.2 mg/l, with a median concentration of 1.5 mg/l. Since heterotrophic bacteria require carbon, nitrogen and phosphorus in a ratio of approximately 100:10:1 (C:N:P), organic carbon may often be the growth-limiting nutrient in finished drinking waters. Assimilable organic carbon is the portion of total organic carbon which can be readily utilized by aquatic organisms for growth. Often, the assimilable organic carbon constitutes just a fraction (0.1 to 9.0%) of the total dissolved organic carbon (LeChevallier *et al.*, 1991).

1.3.2.2 Phosphate-limited growth

The conditions of phosphate limitation are common in natural ecosystems. Under phosphate limitation the rate of growth of microorganism is limited by the rate of transport of this nutrient source (Robertson & Button, 1979). These researchers also showed that under phosphate limitation at low growth rates approximately 10% of the amount accumulated leaked from the cells, both in the form of inorganic phosphate and of phosphate-containing metabolites. The composition of the cell envelope of Gram-negative bacteria, in particular the lipid composition of the membranes varies markedly with changes in the physicochemical composition of the growth environment (Ellar, 1978). The work of Minnikin and Abdoirahmzadeh (1974) reported the effect of phosphate limitation on the phospholipid content of marine *Pseudomonas fluorescens*. Lipid extracted from phosphate-sufficient, magnesium-limited chemostat cultures of the organism contained phosphatidyl-ethanolamine, phosphatidyl-glycerol and diphosphatidyl-glycerol. However, under phosphate limitation at a dilution rate of 0.2 h^{-1} , no traces of phospholipids were detected in lipid extracts. These were completely replaced by ornithine-containing lipids and an acidic glycolipid,

indicating that in this organism acidic and zwitterionic lipids totally lacking phosphate may take the place of phospholipids in the cytoplasmic membrane under phosphate limitation.

1.3.2.3 Nitrogen-limited growth

Nitrogen is an essential nutrient and, depending on the properties of particular organisms, may be supplied in the form of organic nitrogen compounds, ammonia, nitrate or molecular nitrogen. Ammonia is the preferred nitrogen source for many aquatic microorganisms. Its concentration in fresh and marine waters is usually very low, approximately a few parts per million. However in aquatic sediments the concentration of ammonia can be higher and this usually diffuses into the water body (Cole & Brown, 1980). The biodegradation of organic materials settling out from the water column is often considered to be the source of much of this ammonia (Rosenberg & Young, 1974). Many organisms are able to utilize ammonia, which is mainly incorporated into glutamate or glutamine, and these compounds in turn serve as the main precursors for the synthesis of other cellular organic nitrogen compounds (Gottschalk, 1979). In many Gram-negative bacteria, two pathways lead to the synthesis of glutamate from ammonia; by glutamate dehydrogenase and by the glutamine synthetase/glutamate synthase pathway (Jansen *et al.*, 1980). In microorganisms, uptake of ammonia from the environment is generally thought to be mediated by facilitated diffusion, but little is known about the way in which the properties of this ammonia transport system change in relation to changes in the external concentration of ammonia during growth of the organisms. In contrast the adaptation of cytoplasmic ammonia-assimilating enzyme systems to environmental ammonia concentrations is well documented (Harder & Dijkhuizen, 1983). The concentration of naturally occurring amino acids is very low in aquatic environments. Most bacteria can synthesize all twenty amino acids necessary for protein biosynthesis utilizing

inorganic ammonium salts as the nitrogen source. The intracellular levels of most amino acids are carefully controlled to meet the varying demands by the normal growth cycle of the cells. To control the intracellular levels the cell must balance the processes that lead to increases in intracellular levels such as biosynthesis with those that decrease the level such as metabolism or protein synthesis. Since transport activities alter the cellular level of amino acids it is important to the cell to have a way of regulating them (Oxender, 1980).

In general the biosynthesis of amino acids is strictly controlled by mechanisms, such as end-product regulation and organisms do not normally overproduce amino acids. These facts imply that the control mechanisms are physiologically effective and also that the bacterial envelope forms a highly impermeable barrier for the free amino acids which are synthesized endogenously. On the other hand, bacteria can also utilize amino acids from the medium, by taking them up actively (Kepes & Cohen, 1962). The transport and accumulation of amino acids into cells are catalysed by functionally specific transport systems which are located in the cytoplasmic membrane. In a broad sense, transport systems serve dual roles in cell physiology: one as chemical sensing systems for detecting nutritional information in the medium and the other as functional barriers for maintaining pool amino acids in the cells (Anraku, 1975).

Several studies have indicated that the cellular levels of free amino acids rise when the growth of Gram-negative bacteria is interrupted. Britten and McClure (1962) found that, when *Escherichia coli* were starved for glucose or nitrogen, the free amino acid content was maintained for several hours. Mandelstam (1960) reported that the total amino acid pool of *E. coli* increased when a source of nitrogen was removed. Ames (1964) reported similar results for *Salmonella typhimurium*. These results may be accounted for in part by the fact that organisms, such as *E. coli* and *S. typhimurium*, do not effectively utilize amino acids as nitrogen sources. Therefore with an increase in protein breakdown without a concomitant increase in protein synthesis they will lead to an increase in free

amino acids. These organisms also underwent a rapid and almost complete loss of internal amino acids when the external carbon or nitrogen source was exhausted (Kay & Gronlund, 1971).

1.3.3 Environmental factors affecting the survival of micro-organisms in natural waters

Several physical and chemical factors have been considered to influence the survival of bacteria in aquatic systems. The factors considered have been water temperature, predation, solar radiation, nutrient deficiency, sedimentation, heavy metals, pH and salinity. Also the effect of the combined interaction or synergistic relationship between these factors on bacterial survival cannot be ignored.

1.3.3.1 Water temperature

Temperature is an important factor influencing the survival of bacteria in aquatic environments. The studies of fresh and estuarine waters in north-eastern America clearly indicated a seasonal cycle in the isolation rates of *A. hydrophila* which was attributed to temperature variation (Rippey & Cabelli, 1980; Kaper *et al.*, 1981; Burke *et al.*, 1984a). The population size of *A. hydrophila* was significantly affected by water temperature. Counts were highest in the summer months and lowest in the winter. Cavari *et al.* (1981) suggested that the significant decrease in *Aeromonas* density in river water during the cold months of the year could be explained by the low metabolic activity of *Aeromonas* at low temperatures. Similarly Barcina *et al.* (1986) showed, in river waters, that *E. coli* survived longer at 10°C than at 28°C, and suggested that survival was due to the lower metabolic activity at 10°C than 28°C. Meynell (1958) suggested that cell injury due to suboptimal temperatures was due to interference with the permeability control mechanisms. On the other hand, in south-eastern America the highest

densities of *A. hydrophila* were encountered in the spring followed by a decline during the summer then a resurgence in autumn (Hazen, 1983). In the Gomati(India) river a similar seasonal cycle was observed, the highest isolation rates of *Aeromonas* occurred in late winter followed by a progressive decline in density during the summer and the monsoon season (Palhak *et al.*, 1988). It appears that in the tropics and subtropics high water temperatures in the summer have an adverse effect on *Aeromonas* densities and that a failure to see a resurgence of the count when the temperature decreases might be attributed to the decrease in the population occurring as a result of the flushing effect of the heavy monsoon rains. Other similar temperature-dependent variations in *Aeromonas* counts were observed in water thermally polluted by a nuclear reactor and in water exhibiting gradients resulting from natural geothermal effluents (Hazen & Fliermans, 1979). Flint (1987) showed that although the survival of *Escherichia coli* was a function of temperature with the longest survival times being recorded at 4°C and the shortest at 37°C, the presence or absence of the aquatic microflora was of more importance. In the total absence of any indigenous microorganisms *E. coli* survival with no decline in viable count for more than 260 days at 25°C and below in laboratory experiments.

1.3.3.2 Predation

Enzinger and Cooper (1976) emphasized the influence of predatory microorganisms, especially the protozoa, on removing *E. coli* from estuarine waters. In their study, *E. coli* disappeared most quickly from samples with high numbers of ciliates present and there was a much slower decline in numbers in samples with no protozoa (removed by filtration through Whatman No.1 filter paper) but with normal numbers of bacteria present. McCambridge and McMeekin (1981) reported that predatory microorganisms played a minor role in *E. coli* removal in estuarine water. *E. coli* survival was enhanced protozoa were inhibited by the

antibiotic, cycloheximide, but was not as long as survival in autoclaved water, suggesting that the difference was due to predation by bacteria rather than competition or predation by bacteriophage. In laboratory studies using river water, Flint (1987) showed little difference in survival times of *E. coli* in untreated and Whatman-filtered samples although the former sample contained protozoa. Autoclaving the water prolonged survival suggesting that either competition for nutrients or phage predation was responsible for the difference in survival times. There was also a difference between survival times in autoclaved sterile river water and Millipore filter-sterilized river water. This difference was suggested by Flint (1987) to be due to phage particles passing through the Millipore filter or due to the presence of ultramicrobacteria in the filtered samples which ultimately became competitors for nutrients or were predators.

1.3.3.3 Solar radiation

Hollaender (1943) showed that visible and near-visible light were lethal to *E. coli* and that the effect was increased in starvation medium. *E. coli* decay in sea water was principally the result of photo-oxidative cell damage enhanced by the simultaneous inactivation of both the energy-producing mechanisms and the protection mechanisms of the cell. The catalase enzyme system was shown to be inactivated by solar radiation leading to death due to hydrogen peroxide build up (Kapuscincki & Mitchell, 1981). Other investigators have suggested that the effects of solar radiation are enhanced by the presence of protozoan predators which presumably remove injured bacteria from the samples (McCambridge & McMeekin, 1981; Barcina *et al.*, 1986). Sunlight can penetrate up to 3.3 m in clear sea water but its capacity to inactivate faecal coliforms is reduced dramatically in turbid waters subject to sewage pollution (Gameson & Gould, 1975; Chamberlain & Mitchell, 1978) or containing light-absorbing substances, such as humic and fulvic acids (Davies & Evison, 1991). Under visible light illumination, the enteric

bacteria lose the ability to form colonies on standard bacteriological media, the ability to incorporate substrates, and biosynthetic processes become progressively inactivated (Barcina *et al.*, 1990).

1.3.3.4 Nutrient deficiency

Natural ecosystems are frequently depleted of one or more of the nutrients essential for vigorous metabolic activity of both the autochthonous microbial populations and non-indigenous microorganisms. Hence microbial growth in natural environments is nearly always nutrient limited and the most common environmental stress imposed on microorganisms is starvation (Tempest & Neijssel, 1981). The growth of *Aeromonas* seems to be directly related to the availability of nutrients, such as assimilable carbon, nitrogen and phosphates, under optimal temperature. *Aeromonas* can multiply in natural aquatic habitats provided the temperature is adequate and growth is aided by the presence of organic matter (Rippey & Cabelli, 1985). Similar effects were shown by Van der Kooij *et al.* (1980) in tap water where these microorganisms could grow with the addition of very small quantities of substrates.

In river water, Hendricks (1972) showed that *E.coli* could grow in waters supplemented with sewage effluent and that the more grossly polluted the site the more growth occurred. Flint (1987) showed essentially similar results with there being longer survival at a sewage polluted river site than at a nutrient impoverished one. In fresh water habitats subject to differing levels of faecal pollution the higher concentrations of *Aeromonas* were found in the more polluted zones (Araujo *et al.*, 1989). Van der Kooij and Hijnen (1988), who determined the nature and the growth-promoting properties of several organic compounds which might serve as growth substrates in domestic water, suggested that biomass components, such as amino acids and long-chain fatty acids, can promote multiplication of *Aeromonas* in water distribution systems at concentrations as low as a few micrograms per litre.

It was shown, in marine waters, that *Aeromonas* could survive and multiply by an outfall of sewage and organic matter from terrestrial effluents (Araujo *et al.*, 1990). Nutrient deficiency and competition for nutrients can not be separated, as even in the presence of sufficient nutrients to sustain growth, non-indigenous bacteria may be non-competitive for the uptake of essential nutrients. Jannasch (1968) showed that a marine spirillum successfully outcompeted *E. coli* at the low nutrient concentrations expected in natural waters because the saturation constant for carbon source uptake systems was orders of magnitude lower than for *E. coli*. In contrast at high nutrient concentrations *E. coli* outcompeted the spirillum because of its superior growth rate in a non-limited environment. Flint (1987) commented on the effects of eliminating competitive microorganisms in studies on the survival of *E. coli* in river water samples. If the competitive microflora was eliminated by autoclaving, or with lesser success by filter sterilization then *E. coli* could survive on the nutrients in unpolluted river water for at least 260 d.

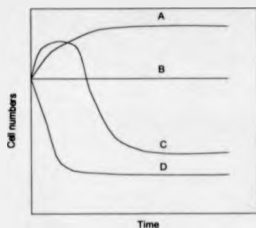
To survive for this period of time with no exogenous nutrient supply, *E. coli*, it was postulated, must enter a dormant state similar to that described by Morita (1982) for a marine *Vibrio* under starvation conditions. Morita described the physiological changes which marine bacteria undergo to prepare themselves for starvation-survival including reductive division which increases the viable count whilst the cells decrease in size, and the switching off of RNA, DNA and protein synthesis (Novitsky & Morita, 1976; Kuruth & Morita, 1983). Flint's (1987) results indicate that *E. coli* could enter such a dormant state in river water but only in the absence of natural microflora of the river. Flint (1987) postulated that in order to become dormant, *E. coli* requires nutrients which are not available due to its poor uptake mechanism if other microorganisms are present.

1.3.3.5 Other factors

Other parameters such as sedimentation, salinity, heavy metals, dissolved oxygen and turbidity have been also considered as having a significant influence on the multiplication and survival of *Aeromonas* and other microorganisms, negatively or positively, in aquatic environments (Gadd & Griffiths, 1978; Hazen, 1983; Ferreira & Lund, 1987).

1.3.4 The patterns of starvation-survival responses in bacteria

Four patterns of starvation-survival are depicted in Fig 1.2 (adapted from Morita, 1985). It was shown that a marine *Vibrio* Ant-300 increased in cell numbers when placed in a starvation medium (Novitsky & Morita, 1976). This pattern was also observed by Kjelleberg *et al.* (1982) with another *Vibrio* sp. and by Kurath and Morita (1983) with a *Pseudomonas* sp., as well as a few freshly isolated, open ocean strains (Amy & Morita, 1983a). This pattern is illustrated by line C in Fig 1.2. The pattern of rapid die-off depicted by line D in Fig 1.2 was shown to be displayed by some of the freshly isolated bacteria of the open ocean (Amy & Morita, 1983a) and probably represents that of most bacteria that grow in energy-rich environments, such as *E. coli*. In natural situations, *E. coli* is released into the environment along with faecal material, hence an energy source is available to the cells. Amy and Morita (1983a) also observed a pattern in which there is an increase in cell numbers followed by the maintenance of a constant level of viable cells (line A in Fig 1.2). Organisms that did not display either a die-off or an increase in cell numbers were shown in line B in Fig 1.2, which was noted with the nitrifying bacteria, and the numbers remained constant until the experiment was terminated (21 weeks) (Morita, 1985).



Key

- A: Oligotrophic open ocean bacteria
- B: Nitrifying bacteria
- C: *Vibrio* Ant-300, *Vibrio* DW1, *Vibrio cholerae*, *Pseudomonas* sp
- D: Most copiotrophic bacteria (e.g., *E. coli*)

Fig. 1.2 Starvation-survival patterns of microorganisms.
(Morita, 1985)

1.3.5 The starvation-survival process of bacteria

Most bacteria in natural waters that have been studied in any detail fit pattern C in Fig 1.2. The first response of bacteria that fit pattern C is to fragment into many small cells. This was observed to happen with marine *Vibrio* Ant-300 (Amy & Morita, 1983a) and *Vibrio* DW1 (Humphrey *et al.*, 1983; Kjelleberg *et al.*, 1983), *Vibrio cholerae* (Baker *et al.*, 1983) and a *Pseudomonas* sp. (Kurath & Morita, 1983). The number of cells produced during the fragmentation process depends on (1) the length of time the organisms have been in the exponential growth phase (the longer period, the larger the number of cells produced) and (2) the initial concentration of cells used to begin the starvation-survival process (Morita, 1985). There are probably other factors, such as type of medium (e.g. carbon-, nitrogen-, or phosphate-limited media) and previous history of the cells.

Nitrogen-limited cells survive better than glucose-limited cells (Jones & Rhodes-Roberts, 1981). There is a large increase in the number of cells due to fragmentation, and Baker *et al.*(1983) reported an increase of 2.5 orders of magnitude within 3 days when *Vibrio cholerae* was placed in nutrient-free artificial sea water. During the fragmentation phase, there can be an intense metabolic activity, as indicated by the oxygen uptake measurements with a *Vibrio* sp. designated as *Vibrio* DW1 (Kjelleberg *et al.*,1983).

Starvation-induced ultramicrocells showed an enhanced rate of adhesion, whereas early log, late log and stationary phase cells showed very little tendency to attach to surfaces (Dawson *et al.*,1981). Humphrey *et al.*(1983) examined various marine isolates during starvation and found that 12 rod-shaped hydrophilic bacteria decreased in size more rapidly at the solid-surface than in the liquid phase, whereas, the 3 rod-shaped hydrophobic bacteria became smaller more rapidly in the liquid phase. They also reported that the starvation of copiotrophs appears to be a two-step process involving a period of rapid size reduction followed by a phase of long-term starvation. Dawson *et al.*(1981) proposed that adhesion of starved cells to surfaces may be a strategy for long-term survival of copiotrophic bacteria. The metabolism of protein occurred during the starvation process and ceased after 2 to 3 weeks (Amy *et al.*,1983a). ATP content, endogenous respiration rate, uptake rates and percentage respiration of exogenous glucose and glutamate stabilized after 18 to 25 days of starvation of a *Pseudomonas* sp. (Kurath & Morita, 1983). When protein fingerprints of starved cells were compared to non-starved cells, some protein spots disappeared, whereas others were seen only during starvation. These starvation-inducible proteins were reported to be the most essential for long-term survival (Reeve *et al.*,1984a,b; Nystrom *et al.*1990b).

1.3.6 Occurrence of ultramicrobacteria

Oppenheimer (1952) observed marine bacteria which pass through 0.45 μm Millipore filters. Ander and Heffernan (1965), using a double filtration method, isolated bacteria from sea water, which could pass through 0.45 μm filters but were retained on 0.22 μm filters. These were subsequently identified as *Spirillum*, *Leucothrix*, *Flavobacterium*, *Cytophaga*, and *Vibrio* sp.

It was also known that low nutrient levels and continued bacterial cell multiplication results in ultramicrocells (Henrici, 1928; Rahn, 1932). More recently, in 1963, Harrison and Lawrence described starvation-resistant mutants of *Aerobacter aerogenes* that were distinctly smaller than the wild type, and Novitsky and Morita (1976) reported that the formation of small cells by starvation could be a response to conditions of prolonged nutrient deprivation. In 1981, Torrella and Morita characterized a form of bacteria which they named "ultramicrobacteria". These were defined as small in size, i.e., less than 0.3 μm in diameter, demonstrating slow growth, and which did not significantly increase in size when inoculated onto a nutrient-rich agar medium. For many of these isolates, biochemical characterization was not performed due to lack of sufficient cellular biomass. The need for minimal nutrient concentrations and a prolonged incubation time appears to be extremely important for recovering ultramicrobacteria (Tabor *et al.*, 1981). Torrella and Morita (1981) also expressed the view that the laboratory concentrations of nutrients employed in their study were inappropriately rich, because it was hypothesized, isolates were conditioned to extreme nutrient limitation. MacDonnell and Hood (1982) recovered minute spherical bacteria that appeared to require conditioning to higher nutrient concentration before the higher concentration of organic matter could be utilized as a substrate for growth. The response elicited by the organisms to increased nutrient concentration was an increase in cell volume and in growth rate, with eventual formation of visible colonies. The failure of ultramicrobacteria or other

native environmental isolates to grow immediately on rich media may be related to substrate-accelerated death, as speculated by Postgate and Hunter (1962). Under laboratory conditions, a population growing under limited substrate conditions exhibits rapid cessation of growth when presented with that substrate under starvation conditions. However the phenomenon of substrate-accelerated death has usually been demonstrated by using relatively large concentrations of a single nutrient, such as glucose, glycerol, or ribose (Postgate & Hunter, 1964; Calcott & Postgate, 1972). Roszak and Colwell (1987) concluded that the ultramicrobacteria are representative of the autochthonous bacterial communities in the estuarine and marine environment. The ultramicrobacteria are, undoubtedly, a portion of the bacteria that are not recovered by standard culture methods and may represent an early strategy in a linear progression of survival mechanisms

1.3.7 Viability and detection

Valentine and Bradfield (1954) proposed the term "viable" to describe cells capable of multiplying and forming colonies, but suggested the term "live" for cells showing other signs of viability, such as respiration, even if the cells were unable to divide under the prevailing conditions. Later experiments utilizing radiolabeled substrates to measure heterotrophic uptake and respiration confirmed the existence of such living populations of cells (Meyer-Reil, 1978). Kurath and Morita (1983) referred to cells as being viable only so long as they demonstrated the capacity to reproduce on an agar medium suitable for growth of the organism. They were described as "nonviable" once the cells lost the ability to form a colony. While applying this definition, Kurath and Morita (1983) nevertheless concluded that the subpopulation of cells that was actively respiring existed at a concentration 10-fold greater than the number of "viable" cells (by their definition). Similarly, Hoppe (1976) concluded that bacteriological methods based on the total number of actively metabolizing bacteria should be used. Postgate and Hunter (1962)

described dead bacteria as those that did not divide conceding however that the other, nondividing bacteria may be in some sense "alive", because they retained their osmotic barriers after "death". Death in this case was defined as the lack of cell multiplication.

Most of the experiments carried out to quantify bacteria have been aimed at measuring viability of bacterial populations. Often these studies were done after application of "stress" or induction of "injury" meaning the imposition of conditions, including heat, cold, drying, pressure, salinity, chemicals, or radiation. Much of this research was reviewed by Andrew and Russell (1984). Two strategies for survival below certain threshold concentrations of substrate, by Jannasch (1967), were observed: (i) the ability to grow at low substrate concentrations and (ii) the ability to become temporarily inactive (nonculturable) but to survive. It can be concluded that living, metabolically active, bacterial populations exist that do not form colonies on agar plates in the laboratory and therefore exhibit no "viable count" (Roszak & Colwell, 1987). Methods which have been employed to isolate and detect cells that are viable but unable to multiply are illustrated in Table 1.4 (from Roszak & Colwell, 1987).

1.3.8 Definition of viable, dormant and viable but non-culturable cell

Throughout this thesis the definition of viable, dormant and viable but non-culturable cells are as follows;

- 1) Viable cells. There are capable of growth, respiration and other metabolic functions and can grow on selective and non-selective media.
- 2) Dormant cells. There are not capable of growth on selective and non-selective media. They may be recovered by resuscitation in liquid media. They have detectable respiration or metabolic activity.
- 3) Viable but non-culturable cells. There are not capable of growth on selective and non-selective media. They can be recovered by passage through a host and

occasionally by resuscination in liquid media with low nutrient concentration. They are capable of respiration at low levels which may be detected using microscopy.

Table 1.4 Methods described for differentiating living from dead bacterial cells (from Roszak & Colwell, 1987)

Method	Protocol
Differential staining for microscopic observation	<p>"Old" vesuvium solution added to leukocyte exudate containing bacteria (live cells, no change; dead cells stain brown)</p> <p>Neutral red stain added to bacterial solutions (live cells, no change; dead cells stain red)</p> <p>Eosin-methylene blue stain added to wet mount (cyanophilic live cells stain blue; eosinophilic dead cells stain red)</p> <p>Bacterial suspension added to neutral red and methylene blue film on microscope slide (live cells stain violet red; dead cells stain blue)</p> <p>Dried and fixed bacteria smear stained with carbol fuchsin and methylene blue solution (live cells stain blue; dead cells stain red)</p> <p>As above, but with spore suspensions (live spores do not stain; dead spores stain blue)</p> <p>Dried and fixed bacterial smear stained 5 min with methylene blue, rinsed, stained 5 to 10 sec with 1:10 carbol-fuchsin (live cells stain blue; dead cells stain red)</p> <p>Methylene blue solution applied to yeast cell preparation (no change in reproducing cells; nonreproducing cells stain blue)</p> <p>Neutral red added to bacterial suspension (live cells remain colorless; dead cells stain red)</p> <p>Congo red applied as negative stain to bacterial suspensions (live cells remain colorless; dead cells stain red)</p> <p>Congo red and methylene blue added to fermenting cultures (live cells remain unchanged; nonreproducing, nonfermenting cells take up stain)</p> <p>Gram stain applied to Gram-positive cells (live cells stain blue; dead cells stain red)</p> <p>Hanging blocks of agar inoculated with bacterial suspension and observed microscopically for multiplication for four generations</p> <p>Individual cell multiplication on solid medium followed by photomicrography</p> <p>Growing microorganisms examined microscopically by oblique incident illumination in a moist chamber on solid nutrient surface</p> <p>Culture chamber containing cellophane membrane inoculated with individual organisms observed for multiplication</p>

(continued on next page)

Table 1.4 - Continued

Method	Protocol
	Graticulated cellophane membrane inoculated with bacterial culture; microscopic counting of organisms before and after a short incubation period
	For orientation, Formvar grid replicas inoculated with bacteria and periodically observed microscopically for multiplication
	Microcolony formation on membrane filters of small cells observed microscopically
	Organisms dispersed in thin agar films suspended in wire loops immersed in liquid medium; after various incubation periods films are stained and observed microscopically on slides for number of bacteria per colony
	Short-term incubation of bacteria on agar films followed by differentiation by microscopic counting of number of microcolonies per number of total bacteria
	Agar films on blotting paper in chambers inoculated with bacterial suspensions and incubated in 1 to 6 h; phase-contrast microscopic observation of growth of viable and moribund cells
	Cellophane membrane inoculated with bacteria in a continuous flow through chamber containing liquid synthetic medium observed microscopically for seven generations
	Microculture, following clones of antibiotic-treated cells for inhibition of multiplication
	Agar blocks containing bacterial suspensions observed by time-lapse, phase-contrast photomicrography for microcolony formation
	Agar films contained in metal rings on microscope slides inoculated with concentrated fresh water sample, incubated, and observed microscopically for microcolonies and single cells
Refractive index	Gelatin solutions abolish light scattering by dead cells, leaving live cells visible
Optical effect	Optical density of a suspension of live bacteria is greater in a saline solution than in distilled water; dead bacteria show no change
	Optical density is 25% greater for live organisms in saline than in distilled water, whereas killed organisms show no effect, mixtures show optical effect in proportion to % live organisms from +25% to -10% of optical density in water; used as a measure of intact osmotic barrier required for living organisms
Constituent leakage	Dead bacteria in culture leak nucleotides; measure labeled nucleotides in culture to estimate amount of dead cells
Division inhibition	Medium containing 3% urea promoted growth but inhibits division of bacteria; electron microscopic observation differentiates growing from dead bacteria
	6-h incubation of bacteria in yeast and nalidixic acid promotes growth while inhibiting cell division in gram-negative bacteria; microscopic counts after incubation of acridine orange-stained preparations differentiate elongated growing cells from short nongrowing cells

(continued on next page)

Table 1.4 - Continued

Method	Protocol
	As above, but various Casamino acids, trypsin, glutamate, and glucose as growth substrates
	Kogure method with staining by fluorescent antibody for specific population enumeration
Respiration	Microscopic observation of bacteria treated with triphenyltetrazolium for reduced products indicative of dehydrogenase activity
	Electron microscopic observation of bacteria treated with tetranitroblue tetrazolium for reduction products indicative of dehydrogenase activity as criterion for viable cell enumeration
	Reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan results in an accumulation of dark red spots which attain size and degree of optical density to allow observation by light microscopy (Respiring bacteria contain spots; nonrespiring cells do not)
	As above, but counterstained with malachite green for additional contrast
Microautoradiograph -y	$[^3\text{H}]\text{glucose}$, $[^3\text{H}]\text{acetate}$; incubation of bacterial sample with radiolabeled compound and eventual filtration of samples, exposure of filter to photographic film, development of film, microscopic viewing of film for decay products of radioactive compounds identified by darkened grains on film
	$[^3\text{H}]\text{glucose}$, $[^3\text{H}]\text{thymidine}$; film emulsion on slides, cells stained with methylene blue
	Scanning electronic microscopic observation of bacteria on detritus after incubation with radiolabeled $[^3\text{H}]\text{glucose}$ and $[^3\text{H}]\text{acetate}$
	$^{32}\text{P}\text{O}_4$; radiographic films microscopically observed and counted for number of spots per sample volume
	$\text{NaH}^{14}\text{CO}_2$ and immunofluorescence staining by fluorescent antibody combined to identify organisms containing radioactivity
	^3H -amino acid mixture, X-ray film; count spots per ml of sample
	$[^3\text{H}]\text{acetate}$, $[^3\text{H}]\text{glucose}$, $[^3\text{H}]\text{leucine}$; stripping film; filter stained and viewed separately for placement of cells
	$[^3\text{H}]\text{glucose}$; film emulsion on slides; acridine orange staining of cells
	$[^3\text{H}]\text{acetic acid}$, ^3H -amino acid, $[^3\text{H}]\text{thymidine}$; acridine orange staining of cells on film emulsion, removal of filter, observation of cells and developed grains in same plane of focus

The aims of this project were;

1. To investigate the physiological and morphological responses of *A. hydrophila* under various stressful environmental conditions, such as starvation, in lake water microcosms.
2. To determine the viability, respiration ability and changes in cell size of *A. hydrophila* under different stresses, nutrient amendments and organic compounds released from other microcosms.
3. To assess the interrelationship between cell survival and the production of scavenging enzymes, such as phosphatase and extracellular protease, in lake water microcosms.
4. To determine the occurrence of gene transfer under biotic and abiotic conditions.
5. To determine the alterations in protein fingerprints which occurred in whole cells and periplasmic space under starvation and other stresses, such as osmotic, oxidative and ethanol stresses and heat shock.
6. Finally to identify alkaline phosphatase, one of the key enzymes in response to stress, on Two-dimensional gel electrophoresis separations.

Chapter 2

Materials and Methods

2.1 Strains

Aeromonas hydrophila was used throughout this study. It was subcultured every two weeks onto nutrient agar plates incubated at 30°C overnight and then stored at 4°C. The organism was originally isolated from the River Sowe, Coventry and characterised by Dr K. Flint, University of Warwick (the details of the biochemical characteristics and adhesive properties of this organism can be found in Neilson, 1991).

Flavobacterium B14 was obtained from Dr P. Hayes, University of Leeds (Hayes, 1977). The organism was routinely subcultured every 4 weeks on nutrient agar, incubated at 30°C for 48h then stored at 4°C until required. *Escherichia coli* R1 drd-19 (*E. coli* K12 with the transfer-derepressed plasmid R1-19 of the F incompatibility group), *Escherichia coli* 2366 (*E. coli* K12 with the transfer-derepressed plasmid R144-3 of the incompatibility group), *Escherichia coli* 2599 (*E. coli* J5 with the transfer-derepressed plasmid R144-3 of the incompatibility group), and *Escherichia coli* nal-R (nalidixic acid-resistant mutant of *E. coli* K12) were obtained from Dr K. Flint's culture collection at Warwick University. Both strains were originally obtained from Dr E. Meynell's collection, University of Kent at Canterbury.

Anacystis nidulans, *Chlorella vulgaris*, *Scenedesmus quadricauda* and *Chlamydomonas reinhardtii* were obtained from culture collections. These organisms were cultured in Bold's basal medium in the presence of fluorescent light in growth cabinet at 30°C then stored at room temperature.

2.2 Media

2.2.1 Nutrient agar

Nutrient agar (Oxoid) was used for viable counts in all experiments and for routine maintenance of the stock cultures. Nutrient agar plates were prepared as per the manufacturer's instructions.

2.2.2 Nutrient broth

Nutrient broth (Oxoid) was used for the routine culturing of bacteria to prepare the inocula for the experiments. Nutrient broth was prepared as per the manufacturer's instructions and stored in sterile 100 ml aliquots in 250 ml Erlenmeyer flasks.

2.2.3 Bold's basal medium

Bold's basal medium (Bischoff & Bold, 1963) was used for culturing of the algae employed in the dialysis bag experiments. Six sterile stock solutions were employed, each containing one of the following salts in the concentration listed :

NaNO_3	10 g
K_2HPO_4	3 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1 g
KH_2PO_4	7 g
$\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$	3 g
NaCl	1 g

1 ml of each stock solution and 0.1 ml of each of the 4 sterile stock trace element solutions were added to 94 ml of sterile distilled water in a 250 ml Erlenmeyer flask. The stock element solutions were made as follows :

1. 50 g EDTA and 31 g KOH dissolved in 1 l H_2O .
2. 5 g $FeSO_4 \cdot 7H_2O$ dissolved in 1 l distilled water containing 0.1 % by volume of H_2SO_4 .
3. 11.5 g H_3BO_3 dissolved in 1 l H_2O
4. 9 g $ZnSO_4 \cdot 7H_2O$, 1.5 g $MnCl_2 \cdot 4H_2O$, 0.7 g $NaMoO_4$, 1.5 g $CuSO_4 \cdot 5H_2O$ and 0.5 g $Co(NO_3)_2 \cdot 6H_2O$ all dissolved together in 1 l H_2O .

All stock solutions were sterilised at $121^\circ C$ for 15 min.

2.2.4 Quarter-Strength Ringers Solution

Quarter-Strength Ringers Solution was used for all serial dilutions. This was prepared as follows :

Sodium chloride	2.25 g
Calcium chloride	0.12 g
Sodium hydrogen carbonate	0.05 g
Potassium chloride	0.105 g
Distilled water	1 l

This was dispensed in 200 ml volumes, autoclaved at $121^\circ C$ for 15 min and stored at $4^\circ C$ until use.

2.2.5 Minimal medium

Minimal salts growth medium was used for growth of cells for many of the enzyme experiments. The medium was made up from sterile stock solutions as follows :

0.1 M Tris-HCl buffer, pH 7.2	500 mls
MgSO ₄ ·7H ₂ O (10 g/l)	10 mls
(NH ₄) ₂ SO ₄ (10 g/l)	100 mls
CaCl ₂ (1 g/l)	100 mls
FeSO ₄ ·7H ₂ O (0.05 g/l)	10 mls
Trace element solution	2.5 mls

This volume was adjusted to 800 ml and dispensed in 80 ml volumes to 250 ml Erlenmeyer flasks. After autoclaving at 121°C for 15 min, carbon and phosphorus sources were added as follows :

Carbon source : 10 mls of 5 g-C/l solutions of the appropriate carbon source were added. Carbon sources were sterilised by autoclaving at 121°C for 15 min.

Phosphorus source : 10 mls of 1 g PO₄/l solution (1.432 g KH₂PO₄/l), sterilised by autoclaving at 121°C for 15 min. This gave a final phosphate concentration of 100 mg PO₄/l. This is referred to as high phosphate medium. Low phosphate medium contained a final concentration of 2 mg PO₄/l. The trace element solution was that of Kelly and Clarke (1962) made up as follows :

(NH ₄) ₂ FeSO ₄ ·6H ₂ O	232 mg
H ₃ BO ₃	464 mg
CoSO ₄ ·7H ₂ O	191 mg
CuSO ₄ ·5H ₂ O	16 mg
MnSO ₄ ·4H ₂ O	16 mg
(NH ₄) ₆ Mo ₇ O ₂₄ ·H ₂ O	44 mg
ZnSO ₄ ·7H ₂ O	348 mg

The trace elements were dissolved in 1 l distilled water and 1 M HCl added dropwise until the solution cleared. In some cases 5 mls of L-glutamic acid or L-proline (2.5 g-N/l solutions) were used instead of ammonium sulphate

as the nitrogen source. All chemicals were obtained from Fisons or BDH and were of the highest purity available.

2.3 Water samples

Lake water samples were collected in 1 l sterile glass bottles from Tocil lake in University of Warwick (OS ref 303756). This is a man-made lake fed by spring water and populated by wild fowl. Lake water samples were filtered through Whatman-No.1 filter paper to remove suspended particulate matter. This is referred to as Whatman-filtered water. 100 ml volumes were added to 250 ml sterile Erlenmeyer flasks for the experiments. Filtered water was autoclaved at 121 °C for 15 min in 100 ml volumes in 250 ml Erlenmeyer flasks to produce Filtered-autoclaved lake water.

2.4 Supplements

In some instances supplements or amendments were added to the water samples. In all cases, these were added aseptically to the sterile water samples.

2.4.1 Synthetic sewage

In the experiments where synthetic sewage was added to the lake water, various concentrations, as required, of the following mixture were added to 100 mls lake water.

Peptone (Oxoid)	0.3 g
Yeast extract (Oxoid)	0.2 g
Urea	0.05 g
$(\text{NH}_4)_2\text{SO}_4$	1.0 g

KH_2PO_4	0.2 g
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1 mg
Trace elements	5 ml

These were dissolved in distilled water (1 l) and sterilised by autoclaving at 121°C for 15 min. The trace element solution used was as described in 2.2.5. This synthetic sewage has a chemical oxygen demand of 350 mg/l.

2.4.2 Carbon sources amendments

Carbon sources were prepared in distilled water to give a stock solution containing 5 g-C/l. These were added at different concentrations to the lake water samples. All carbon sources were sterilised by autoclaving at 121°C for 15 min. The carbon sources used were :

Glucose (1.25 % w/v, BDH)
 Lactose (1.25 % w/v, Fisons)
 Sodium acetate (1.71 % w/v, Fisons)
 Sodium succinate (2.81 % w/v, Fisons)
 Glycerol (1.14 % w/v, Prolabo)

2.4.3 Phosphorus amendments

KH_2PO_4 (BDH) was used as a phosphorus source amendment. A stock solution containing 1 g PO_4 /l (1.432 g KH_2PO_4 /l) was prepared in distilled water and sterilised at 121°C for 15 min.

2.4.4 Nitrogen source amendments

Several nitrogen sources were used as lake water amendments. These were casein(Sigma), urea(Fisons), sodium nitrate(BDH), and ammonium

sulphate(BDH). The concentrations needed were prepared in distilled water then sterilised by autoclaving at 121°C for 15 min.

2.4.5 Amino acid amendments

Amino acids were prepared in distilled water and sterilised by autoclaving at 121°C for 15 min. The amino acids used were :

L-arginine (L-arg)	L-valine (L-val)
L-tryptophan (L-trp)	L-cysteine (L-cys)
L-tyrosine (L-tyr)	L-methionine (L-met)
L-serine (L-ser)	L-isoleucine (L-ile)
L-threonine (L-thr)	L-proline (L-pro)
L-histidine (L-his)	L-leucine (L-leu)
L-alanine(L-ala)	L-lysine (L-lys)
L-asparagine(L-asn)	L-glutamate (L-glu)
L-phenylalanine (L-phe)	Glycine (gly)
L-aspartate (L-asp)	

Various concentrations were prepared and used as supplements. All amino acids were obtained from Sigma except glycine and L-leucine which were obtained from Fisons and BDH respectively.

2.4.6 Sodium chloride amendments

Sodium chloride was added to the lake water in flasks prior to sterilisation to give the required concentrations then the flasks were autoclaved at 121°C for 15 min.

2.4.7 Osmoprotectants

Betaine (Sigma) was used as an osmoprotectant. 0.1 M betaine was prepared in sterile distilled water and sterilised by filtration through 0.22 μ m cellulose nitrate filters (Sartorius). The stock solution was stored at 4°C until use.

2.4.8 TTA buffer

TTA (TES-Tris-Acetic acid) buffer was used for pH profiles in phosphatase and exoprotease assays. 1M TTA buffer was prepared as follows:

<i>N</i> -tris(hydroxymethyl)methyl-2-amino ethanesulphonic acid (TES)	7.64 g
Tris-(hydroxymethyl)-methylamine	4.06 g
Acetic acid	2.5 ml
Distilled water	100 ml

10 ml of this solution dissolved was diluted to 90 ml of distilled water to make 0.1 M TTA buffer then pHs were adjusted from 4 to 11 using 1 M HCl or 10 N NaOH. These were sterilised by autoclaving at 121°C for 15 min. TES and Tris were obtained from Sigma, U.K.

2.4.9 Other amendments

Cadmium chloride, hydrogen peroxide, and ethanol were used as stress-inducing chemicals. Cadmium chloride was prepared in sterile distilled water and filter-sterilised through 0.22 μ m cellulose nitrate (Sartorius) filters. Hydrogen peroxide and ethanol were used directly and aseptically.

2.5 Dialysis bag experiments

Dialysis bags (pore size, 2.4 nm) sterilised by autoclaving in distilled water at 121°C for 15 min were prepared. Cultures of *Flavobacterium*, *Anacystis*, *Chlorella*, *Scenedesmus* and *Chlamydomonas* were harvested by centrifugation at 5°C then the pellet was resuspended in filtered-autoclaved lake water. Various volumes of each culture were inoculated into sterilised dialysis bag. The bags were suspended in Erlenmeyer flask containing 100 ml filtered-autoclaved lake water aseptically in an air flow cabinet, then survival experiments were conducted as described in 2.6.

2.6 Microcosm survival experiments

A. hydrophila was grown overnight to stationary phase in nutrient broth flasks in a shaking incubator at 30°C. 10 ml of culture were harvested, centrifuged in a sterile tube and resuspended in 10 mls of sterile distilled water. 100 mls of lake water were inoculated with 0.1 ml of this *A. hydrophila* suspension to give an initial concentration of cells of approximately 2×10^6 c.f.u./ml. The flasks were incubated in the dark or under fluorescent light at the appropriate temperature. After shaking the flask, 0.5 ml samples were removed on each sampling occasion and used for viable counts. Stationary phase cells were used in preference to exponential phase cells. Whilst stationary phase cells may have different metabolic characteristics compared to exponential phase cells, these were chosen because the collection of stationary phase cells was considered to be more reproducible.

2.7 Viable counts

Survival of the inoculated *A. hydrophila* was determined by a spread-plate viable count method. 0.5 ml samples were removed from the flasks and serial ten-fold dilutions prepared in quarter-strength Ringers solution. 0.1 ml volumes were plated in triplicate from the appropriate dilutions. Plates were incubated overnight at 30°C. All plates were counted and results were expressed as mean number of colony forming units per ml.

2.8 Epifluorescence microscopy

Epifluorescence microscopy (Zimmerman *et al.*, 1978) was used to examine bacterial total counts, respiring and non-respiring bacteria. Bacterial samples were taken from the flasks and processed immediately as follows.

• Processing of samples

4 ml quantities of each lake water sample were placed into sterilised vials. A 1 ml amount of 0.2 % (w/v, aqueous solution) INT dye (Sigma) was added to each subsample. After mixing carefully the subsamples were kept for 30 min in a 30°C water bath. All aqueous solutions (double distilled water), including the alcoholic solution needed for the following microscopic preparations, were purified by filtration through cellulose nitrate filters (Sartorius, pore size, 0.2 µm) before use.

• Preparation of microscopy

A stock of polycarbonate filters (Nucleopore, 25 mm in diameter and 0.2 µm in pore size), which had been checked before use for uniform wettability, were immersed for at least 1 day in a solution of Sudan Black B (BDH) in 50% ethanol. The final concentration of Sudan Black B was 1:15,000 (Sudan Black

B had to be dissolved in absolute ethanol, which then was diluted to 50% with double distilled water). Before use the filters were thoroughly rinsed in double distilled water. Lake water samples from the microcosms were filtered through polycarbonate filters. A uniform distribution of particles was obtained by supporting the filter with a silver membrane (pore size, 0.8 μm). After filtration, 1 ml of an acridine orange solution (1:10,000 in 6.6 mM phosphate buffer, pH 6.7) was pipetted onto the filter. The contact time was 5 min. The filter was taken off the filtration device, air-dried, and cut into wedges with a razor blade. One wedge was mounted with a drop of immersion oil on a microscope slide and covered with a coverslip. Embedded filter wedges were not stored for longer than 2 h.

• Microscopic examination

The mounted filter was examined by epifluorescence microscopy (Nikon Microscope). The total number of bacteria and the proportion of respiring bacteria were determined within the area of a 5×5 graticulated eyepiece. The time required for sample processing and microscopical examination was less than 45 min. Microscopical examination of each filter preparation (25 areas counted) was performed within 30 min.

2.9 Cell volume distribution analysis

A Coulter Counter model ZBI and Coulter Channelyzer C1000 connected to a BBC microcomputer were used to determine size distributions and total cell count. Cell samples (50-200 μl) were diluted into 20 ml of balanced salt electrolyte (Isoton ; Coulter Electronics Ltd) and profiles obtained using a 30 μm orifice probe. Profiles were stored on floppy disc and printed on a Tandy TRS-80 plotter for comparison. The computer program used included a calibration file which compared the cell-volumes with those of latex particle standards of

known size distribution. Cell counts were taken as the mean of five determinations, using the 30 μ m orifice probe and an amplification setting of 0.5.

2.10 Assay for alkaline phosphatase

A quantitative assay for alkaline phosphatase was carried out using the method of Flint (1974). Assays were performed at 30°C in a mixture containing 0.3 ml of the cell suspension and 2.6 ml of Tris-HCl buffer (pH 9.0, 0.1 M) and 0.1 ml of 5% PNPP (para-nitrophenyl phosphate). The tube was incubated for 4 h. The reaction was then stopped by the addition of 0.1 ml 10 N NaOH. The amount of para-nitrophenol released was determined by measuring the absorbance at a wavelength of 420 nm against a blank derived from a reaction mixture treated identically except that it contained no bacterial cells. The activity of alkaline phosphatase is expressed as micromoles of para-nitrophenol released per hour of reaction time per ml.

2.11 Assay for acid phosphatase

Assays for acid phosphatase were carried out as follows essentially as described in Flint (1974). A mixture which contained 1 ml of bacterial sample and 0.3 ml of 2%- β -glycero-phosphate and 0.7 ml of 0.2 M TTA (Tes-Tris-Acetic acid, pH 5.0) buffer was incubated at 30°C for 24 h. 2.5 ml of phosphate reagent solution was then added. The reagent contained 1 ml of 10% ascorbic acid and 6 ml of 0.42% ammonium molybdate in 1 N H_2SO_4 , both of which were mixed just before use. The final absorbance was read at 880 nm after incubation at 30°C for 1 h.

2.12 Assay for exoprotease

The total protease activity of culture supernatant fluids was measured quantitatively with azocasein (Sigma) as the substrate by the method of Jagger *et al.* (1983). 2 ml bacterial samples were centrifuged to remove the cells, and supernatant fluids were incubated with 1 ml of 1.5% (w/v) azocasein for 4 h at 30°C. The assay was terminated by plunging the tubes into ice and immediately adding 2 ml of 10% (w/v) trichloroacetic acid. After at least 1 h, the precipitated substrate was removed by filtration through 0.22 μ m cellulose nitrate (Sartorius) filters, and the absorbance was measured at 400 nm.

2.13 Conjugal transfer assay

The donor strain, *A. hydrophila* carrying kanamycin-resistant plasmid R1-19 which was originally transferred from *E. coli* R1 *drd*-19, and the recipient strain, *E. coli* nal-R, were grown separately overnight in nutrient broth at 30° or 37°C respectively. They were subsequently inoculated together into mating medium (starved- or nutrient amended filtered autoclaved lake water) at a final concentration of 1.28×10^9 and 1.23×10^9 c.f.u./ml respectively. Conjugal transfer was conducted at different temperatures without shaking for the experimental period.

2.14 Estimation of transfer frequency

The frequency of transfer was calculated as the number of transconjugants per number of surviving recipients.

2.15 Enumeration of donor, recipient and transconjugant bacteria

Before and after incubation, samples from matings were serially diluted in quarter-strength Ringers solution and viable counts were done as described in 2.7. Viable counts of donors and recipients were made on nutrient agar plates supplemented, respectively, with kanamycin (50 μ g/ml) to select donor cells and nalidixic acid (50 μ g/ml) to select recipient cells. Transconjugants were selected and enumerated on nutrient agar plates supplemented with both kanamycin(50 μ g /ml) and nalidixic acid (50 μ g/ml). Nutrient agar plates were incubated overnight at 30°C for donors, and at 37°C for recipients and transconjugants.

2.16 Isolation of plasmid DNA

Plasmid DNA was isolated by the alkline lysis method of Birnboim and Doly (1979).

1. 1.5 ml of the cells were pipetted into an Eppendorf tube , and centrifuged for 1 min in an Eppendorf centrifuge.
2. The medium was removed by aspiration to leave the bacterial pellet as dry as possible.
3. The pellet was resuspended by vortexing in 100 μ l of an ice-cold solution of :

50 mM glucose
10 mM EDTA
25mM Tris-HCl (pH 8.0)

4. This was stored for 5 min at room temperature with the top of the tube open.
5. 200 μ l of a freshly prepared solution of alkaline SDS were added. Alkaline SDS contained :

0.2 N NaOH

1% SDS

The top of the tube was closed and the contents mixed by inverting the tube rapidly two or three times. The tube was stored on ice for 5 min.

6. 150 μ l of an ice-cold solution of potassium acetate (pH 4.8) were added. The solution was made up as follows : To 60 ml of 5 M potassium acetate were added 11.5 ml of glacial acetic acid and 28.5 ml of H₂O. The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. The cap of the tube was closed and the tube vortexed gently in an inverted position for 10 seconds. The tube was then stored on ice for 5 min.

7. The tube was centrifuged for 5 min in an Eppendorf centrifuge at 4°C, and the supernatant transferred to a fresh tube.

8. An equal volume of phenol/chloroform (1:1) was added and mixed by vortexing. After centrifuging for 2 min in an Eppendorf centrifuge, the supernatant was transferred to a fresh tube.

9. Two volumes of ethanol were added at room temperature and mixed by vortexing. The tube was allowed to stand at room temperature for 2 min, then centrifuged for 5 min in an Eppendorf centrifuge at room temperature.

10. The supernatant was removed and the tube stood in an inverted position on a paper towel to allow all of the fluid to drain away.

11. 1 ml of 70% ethanol was added, the tube vortexed briefly and recentrifuged.

12. Again all of the supernatant was removed as in step 10. The pellet was briefly dried in a vacuum desiccator.
13. 50 μ l of TE (pH 8.0) containing DNase-free pancreatic RNase (20 μ g/ml) was added and the tube vortexed briefly. 10 μ l of the solution was used for gel electrophoresis. TE (pH 8.0) contained :

10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)

2.17 Agarose gel electrophoresis

Gel electrophoresis was carried out as recommended by Rochelle *et al.* (1985). The method used was as follows :

1. 0.8% (w/v) agarose (Sigma) was added to a measured quantity of Tris-borate buffer (pH 8.0). Tris-borate buffer contained ;

10.8 g Tris base
5.5 g boric acid
0.93 g EDTA
1 l distilled water

2. The slurry was heated in a boiling-water bath until the agarose dissolved.
3. The solution was cooled to 50°C, and ethidium bromide to a final concentration of 0.5 μ g/ml was added to the solution.
4. The edges of gel-forming tray were sealed with autoclave tape so as to form a mold.
5. The warm agarose solution was poured into the mould and the comb was clamped immediately for making sample wells. 0.5-1.0 mm of agarose between the bottom of the teeth and the base of the gel was maintained so that the sample wells were completely sealed.

6. After the gel was completely set (30-45 minutes at room temperature), the comb and autoclave tape were removed and the gel was mounted in the electrophoresis tank.
7. Electrophoresis buffer was added to cover the gel to a depth of about 1mm.
8. 10 μ l of plasmid DNA sample was mixed with 5 μ l of loading dye (30%, w/v, glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol).
9. This mixture was loaded into the wells of an agarose gel, then the gel was run at 80 V for 3 h.

2.18 Transmission electron microscopy

Samples were examined in the transmission electron microscope by negative staining with phosphotungstic acid in the following manner. A drop of the suspension was placed on a Formvar-coated copper grid (100 segment mesh; Agar Scientific) for 30 sec - 1 min before excess liquid was removed with a strip of filter paper. The grid was allowed to dry and the sample negatively stained by placing a drop of 1% (w/v) phosphotungstic acid (pH 7.0) onto the grid. After a few seconds this was removed with a strip of filter paper. Specimens were examined using a Jeol JEM-100S transmission electron microscope operating at an accelerating voltage of 80 kV. Photographs were taken using Kodak Panatonic film. This was developed in Kodak D-19 developer at 20° C for 3 minutes and fixed in Kodak fix. Prints were made using Kodak Veribrom paper.

2.19 Two-dimensional electrophoresis

This technique, adapted from O'Farrell's (1975) method, separates proteins according to isoelectric point in the first dimension and according to

molecular ratio by SDS-PAGE in the second dimension. These two unrelated parameters give maximal resolution of proteins in complex mixtures (O'Farrell, 1975). A stable pH gradient was formed using commercial ampholines and the proteins electrophoresed to equilibrium until their net charge was zero and migration ceased. The buffers and solutions were prepared as follows :

A. Lysis buffer

5.7 g of urea
0.4 ml of pH 5 to 8 ampholine
0.1 ml of pH 3.5 to 10 ampholine
0.4 ml of Triton X-100
1.0 ml of 2-mercaptoethanol
8.0 ml of glycerol
Distilled water to 20 ml

This buffer was stored as frozen aliquots at 20°C.

B. Sample overlay solution

5.4 g of urea
0.2 ml of pH 5 to 8 ampholine
0.05 ml of pH 3.5 to 10 ampholine
4.0 ml of glycerol
Distilled water to 20 ml

This solution was stored as frozen aliquots at 20°C.

C. Gel overlay solution

4.8 g urea /10 ml (8 M urea)

D. Triton X-100 stock solution

10% (v/v) Triton X-100 in distilled water was stored at 4°C.

E. Overlay agarose

1.0 g of agarose melted in 100 ml of SDS-sample buffer, stored at 4°C.

F. 30% (w/v) acrylamide stock for isoelectric focusing

28.38 g of acrylamide
1.62 g of bisacrylamide
Distilled water to 100 ml

This solution was filtered through Whatman No.1 filter paper and stored at 4°C in a dark bottle.

G. Ammonium persulphate solution

A fresh 10% (w/v) solution was prepared each day.

H. Anode electrode solution ; 0.01 M H_3PO_4

I. Cathode electrode solution ; 0.02 M NaOH

J. Lower gel buffer

1.5 M Tris-HCl, pH 8.8
0.4% SDS (w/v)

This buffer was stored at 4°C.

K. Upper gel buffer

0.5 M Tris-HCl, pH 6.8
0.4% SDS (w/v)

This buffer was stored at 4°C.

L. SDS sample buffer

10% glycerol (w/v)
5% (v/v) 2-mercaptoethanol
2.3% (w/v) SDS
0.625 M Tris-HCl, pH 6.8

This buffer was stored at 4°C

M. 30 % (w/v) acrylamide stock for SDS-PAGE

29.2 g of acrylamide
0.8 g of bisacrylamide
Distilled water to 100 ml

This solution was filtered through Whatman-No.1 filter paper and stored at 4°C in a dark bottle.

N. Running buffer

0.025 M Tris base
0.192 M glycine
0.1% (w/v) SDS

This buffer was adjusted to pH 8.3 with HCl and stored at room temperature.

O. TEMED (N,N,N,N, tetramethyl ethylene diamine)

It was used as supplied.

2.19.1 Sample preparations

The bacterial cells were harvested by centrifugation at 8000 rpm for 5 min using a microcentrifuge and the pellet resuspended in lysis buffer, then left at

room temperature until lysis was complete. All lysed cell preparations were run immediately or stored at 20°C until use.

2.19.2 First dimension

The first dimension isoelectric focusing gels were made in 130 mm × 2.5 mm internal diameter glass tubing. This glass tubing was successively cleaned by soaking in chromic acid, then distilled water, then alcoholic potassium hydroxide and finally in distilled water once again before airdrying. The bottom of the tubes was covered with two layers of para-film and filled to a depth of 10 cm with gel mixture.

Isoelectric focusing gel mixture (10 ml)

- 5.5 g urea
- 0.1 ml of ampholine (pH 3.5-10)
- 0.4 ml of ampholine (pH 5.0-8.0)
- 1.33ml of acrylamide stock
- 2 ml of 10 % Triton X-100
- 1.97 ml of distilled water

This mixture was degassed under vacuum then 10 μ l of 10% (w/v) ammonium persulphate added. The solution was loaded into glass tubes immediately after addition of 7 μ l of TEMED. This solution was overlayed with gel overlay solution and allowed to set for 1-2 hours. This overlay solution was removed and replaced with 20 μ l of lysis buffer and this was overlayed with 20 μ l of water. After allowing to set for a further 1-2 hours the lysis buffer and water were removed. 20 μ l of fresh lysis buffer were added, and the tubes filled with 0.02 M NaOH. The para-film at the bottom of the tubes was removed and the tubes were placed in the electrophoresis tank. The lower chamber was filled with 0.01 M H_3PO_4 and the upper chamber was filled with

0.02 M NaOH. The gels were prerun at 200 V for 15 min, 300 V for 30 min, and 400 V for 30 min. After the prerun the power was turned off and the upper reservoir emptied. The lysis buffer and NaOH solution was removed from the tubes, the samples loaded then overlayed with 10 μ l of sample overlay solution and this was overlayed with 0.02 M NaOH solution. The chamber was refilled with 0.01 M H_3PO_4 and the gels were run at 400 V for 12 hours and then at 800 V for 1 hour. The gels were then extracted into 5 ml of SDS sample buffer and equilibrated on a shaker for 2 hours. They were then loaded onto the second dimension gel or stored at 20°C.

2.19.3 Second dimension

Glass plates used in making the slab gels were 20 \times 20 cm in size. Acrylamide solutions for the resolving gel were prepared as follows (per 50 ml):

- 25 ml of acrylamide stock
- 12.5 ml of distilled water
- 12.5 ml of lower gel buffer

After addition of 40 μ l of ammonium persulphate and 7 μ l of TEMED, the above mixtures were poured between the glass plates up to a level 25 mm below the bottom of the notch and were gently overlayed with 2 ml of distilled water. The mixtures were then allowed to polymerize overnight. The next day the stacking gel mixtures were prepared (per 10 ml):

- 2.5 ml of upper gel buffer
- 1.5 ml of acrylamide stock
- 6 ml of distilled water

After 20 μ l of ammonium sulphate and 2.5 μ l of TEMED were added to this mixture, the distilled water overlay was removed and the chamber was filled with gel mixture to the base of the notch. The mixture was allowed to polymerize for 30 to 60 min after small amount of distilled water was overlayed on it. Distilled water was removed and the surface was blotted with Whatman-No.1 filter paper. The isoelectric focusing gel was straightened on the para-film then transferred by sliding onto the top of the stacking gel. The tube gel was sealed into place with 1.5 ml overlay agarose. About 5 min were allowed for the agarose to set, then the bottom gasket was removed, and the gel was clamped to the tank. Running buffer was added to both the upper and lower reservoir and bubbles were removed from the bottom of the gel using a 10 ml syringe. Gels were run at 20 mA constant current until the stained protein marker reached the bottom of the gel. The running time was about 16 hours. Proteins were visualized on the gels by silver staining (see 2.23.2 "Silver staining").

2.19.4 Measurement of pH gradient

The isoelectric focusing gel was cut into 1 cm sections and placed in vials containing 2 ml of distilled water. These vials were capped and shaken for 5-10 min, then the pH was measured on a pH meter.

2.20 Preparation of periplasmic proteins

Periplasmic proteins were isolated by the EDTA-lysozyme procedure of Morris *et al.* (1974). 50 ml bacterial samples were harvested by centrifugation at 12,000 g and washed in cold buffer (10 mM Tris-HCl pH 7.5, 1mM $MgCl_2$). The cell pellet was resuspended in 0.8 ml of a solution containing 25 % sucrose and 10 mM Tris- HCl (pH 7.5). After the addition of 100 μ l of a solution of lysozyme (5 mg/ml) and 100 μ l of EDTA (20 mM, pH 7.5), the suspension was

incubated for 20 min at room temperature. The supernatant fluid obtained by centrifugation of the suspension at 13,000 g, was used as the periplasmic protein fraction. This fraction was lyophilized then stored at -20°C until use.

2.21 Determination of protein concentration

Protein concentrations were determined using the Coomassie Brilliant Blue G-250 reagent with crystalline bovine serum albumin used as standard (Bradford, 1976). The method used was as followed :

A. Preparation of protein reagent

Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 litre. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

B. Protein assay

To the bacterial sample was added an equivalent volume of 0.1N NaOH and the sample was kept in the water bath at 70°C for 30 min. A dilution series of the protein sample was made up using 0.15 M NaCl and 0.1 ml of protein sample was pipetted into 12 × 100 mm test tube. 5 ml of protein reagent was added to the test tube and the contents mixed either by inversion or vortexing. The absorbance at 595 nm was measured after 2 min in 3 ml cuvettes against a reagent blank prepared from 0.1% of 0.15 M NaCl and 5 ml of protein reagent.

2.22 Gel staining and destaining

2.22.1 Coomassie blue staining

This was used for staining Polyvinylidene difluoride membrane for microsequencing described in 2.25.

2.22.2 Silver staining

The method used to silver stain polyacrylamide gels was based on that of Wray *et al.* (1981). Gels were soaked in 50% (v/v) methanol for 6 hour on a gently shaking platform. The methanol was changed 3 times (every two hours) to ensure removal of Tris and glycine from the acrylamide matrix.

The staining solution solution was prepared as follows.

Solution A : 20% (w/v) silver nitrate (AgNO_3 , Sigma) in double-distilled water.

Solution B : 42 ml of 0.36% NaOH was mixed with 0.8 ml of 35% ammonia solution.

Solution C : 8 ml of solution A was added dropwise to solution B with constant swirling and then made up to 200 ml with double-distilled water.

The gel was transferred to the solution C (the staining solution) for 15 min with constant agitation, then washed in two changes of double-distilled water for 5 min each. Following this, the developing solution was made up as follows.

Solution D : 2.5 ml 1% (w/v) citric acid was mixed with 0.25 ml of 38% formaldehyde. This was made up to 500 ml with double-distilled water.

The gel was soaked in solution D to develop the silver stain for 15 min with agitation until protein spots appeared, and then washed in double-distilled distilled water. The gel was placed in 45 % (v/v) methanol and 10 % (v/v) acetic acid to stop stain development. The gel was then photographed.

2.23 Densitometric scanning of stained gels

The stained wet polyacrylamide gel to be scanned was carefully laid on a glass plate and overlaid with a second glass plate such that no air bubbles were trapped between the glass and gel surfaces. A small volume of water was periodically introduced between the glass plates to prevent the drying of the gel. The glass plates containing the gel were then laid on the mobile carriage of the computing densitometer (Molecular dynamics). Stained gel tracks were scanned for 80 s using wedge 1050. Data were automatically collected and printed using Molecular dynamics microcomputer. Data were analysed using dedicated software (Warwick control systems, UK).

2.24 N-terminal sequence analysis

Two-dimensional proteins were blotted onto Polyvinylidene difluoride membrane for microsequencing. Microsequencing was carried out using an Applied Biosystems Gas phase Sequencer by Dr K. Lilley (Department of Biochemistry, University of Leicester). After Two-dimensional gel run, the blot into Polyvinylidene difluoride membrane was prepared as follows.

1. The gel was incubated for 15 min in blot buffer, and blotted for 2-2.5 h at 250 mA with water cooling. Polyvinylidene difluoride membrane, prewetted in 100% methanol and washed in blot buffer for a few minutes, was used for the blot support.

Blot buffer

Tris	5.814 g
Glycine	2.93 g
Methanol	100 mg
SDS	0.3 g
Distilled distilled water	to 1 l

2. After blot, the Polyvinylidene difluoride membrane was stained in Coomassie blue R-250 (0.1%, w/v) in 50% (v/v) methanol, 5% (v/v) glacial acetic acid and 45% (v/v) distilled water for 10-15 min with shaking.

3. The membrane was destained in 50% (v/v) methanol until protein spots were visible, then transferred to 5-10% glacial acetic acid.

4. The membrane was washed in distilled water and air-dried, then stored in a sealed bag at -20°C until use.

2.25 Identification of alkaline phosphatase

The method used for the identification of alkaline phosphatase was as follows:

Two-dimensional protein gel was soaked in at least three changes of 0.1 M Tris (pH 9.0) and 0.1 M MgCl_2 for 24 h with constant shaking to remove SDS from the acrylamide matrix. The gel was transferred to 50 ml of 0.1 M Tris (pH 9.0) containing 50 mg β -naphthyl phosphate and 2 ml of 0.1 M MgCl_2 . After 10 min shaking, 50 mg Fast Blue B.B was added to the solution. A brown spot appeared where alkaline phosphatase activity was present in the gel. The gel was then placed in 7% (v/v) glacial acetic acid for storage and photography.

2.26 Statistical analysis

All points on graphs were means of data obtained as follows; Plate counts, total direct counts, and measurements on cell volumes and enzyme activities were made by conducting in triplicate ($n=3$). Statistical analysis was carried out using the MINITAB soft ware package (Minitab Statistical Software,

State College, Pa., USA) to obtain Tukey's honestly significant difference (HSD) between means calculated from analysis of variance using the method of Peterson (1985). The calculation for HSD is shown as follows;

Tukey's honestly significant difference (HSD)

$$\text{HSD} = Q_a \sqrt{(\text{m.s.e.} / r)}$$

Where:

Q_a value from Studentized (Q) table; 95% confidence limits.

m.s.e. mean square error.

r sample size (number of replicates).

Chapter 3

The Effect of Environmental Factors on the Survival of *Aeromonas hydrophila* in Lake Water Microcosms

3.1 Introduction

Water is a habitat of a biochemically versatile microflora. To survive in natural waters, bacteria must respond to a variety of environmental variables. Several factors have been suggested to have a significant impact on the survival of bacteria in natural waters. There have been discussed in detail in 1.3.3. Barcina *et al.* (1986) proposed that light was a significant stress affecting bacterial survival in river waters, and the susceptibility of *E. coli* to photoinactivation has been shown (Lessard & Sieburth, 1983). Temperature is also known to have a major influence on the persistence of *E. coli* in natural waters (McFeters & Stuart, 1972). The studies of Flint (1987) suggested further that competition for nutrients and temperature were the major factors influencing *E. coli* survival in fresh water. The lack of persistence in water of some species has been attributed to the presence in water of low molecular weight toxins (Klein & Alexander, 1986). Evidence has also accumulated that small zooplankton may be active in grazing on bacteria in fresh water environments (Sanders & Porter, 1986).

Bacteria introduced into the same waters may differ markedly in their persistence (Liang *et al.*, 1982) and for some species at least, these differences appear to be attributable to their resistance to starvation (Sinclair & Alexander, 1984). Some marine *Vibrios* even maintain their viability in a minimal salts solution for as long as one year (Novitsky & Morita, 1977). Actually these environmental parameters frequently are less than optimal for bacterial growth and often the consequence is "die-off", i.e., the repeated finding that bacteria steadily decrease in numbers upon exposure to the natural environment when enumerated by culture methods and eventually can no longer be recovered by these culture methods (Singleton, 1983). Even in the laboratory bacterial growth cultures are limited by various elements, e.g., phosphorus, magnesium, carbon, or nitrogen, when populations of bacteria in the death (or decline) phase eventually reach an extended steady state with 2 to 4% of the cells remaining viable as measured by

colony forming ability (Ribbons & Dawes, 1963). This latter stage is sometimes referred to as "cryptic growth", the condition when a portion of starved microbial populations dies, releasing products of lysis and leakage that support the growth of surviving bacteria (Postgate, 1976).

Survival was defined by Stevenson (1978) as maintenance of viability under adverse circumstances but less clear is what constitutes "viability" and what constitutes "adverse". There are microorganisms that neither sporulate nor encyst but can be isolated from unfavourable environments. Such microorganisms persist as vegetative cells but use up their energy reserves slowly as a result of lowered metabolic activity (Novitsky & Morita, 1978). The suggestion that the changes which bacteria undergo in response to starvation are associated with the depletion of cellular reserves, with less essential material being scavenged for essential needs, was made by Tabar *et al.* (1981). Indeed autochthonous aquatic bacteria might be expected to possess such capabilities, even though some allochthonous bacteria may also employ similar strategies for survival under conditions encountered in an unfavorable environment (Roszak *et al.*, 1984).

A pragmatic definition of viability of bacteria was offered by Postgate (1969), namely, that viability is the property possessed by that portion of a bacterial population capable of multiplication when provided with optimal conditions for growth. However what constitutes an "optimal environment" is not known for many native aquatic bacterial species and varies significantly with individual populations (Postgate, 1969). In fresh water and marine environments, two basic types of populations of bacteria were found to exist: those that can grow slowly at very low concentrations of nutrients, termed "oligotrophs", and those that grow only at higher concentrations of nutrients but survive low nutrient conditions by a variety of mechanisms, termed "copiotrophs" (Martin & MacLeod, 1984). In the study of the relationship between the number of oligotrophs or copiotrophs in fresh and sea water samples and a number of environmental factors, Yanagita *et al.* (1978) suggested that (a) oligotrophs and copiotrophs responded quite similarly to chemical environmental

factors and (b) the number of these trophic groups remained almost constant at even higher concentrations of chemical substances. In fresh water areas, the number of oligotrophs usually exceeded that of copiotrophs, and limiting substances for the size of these bacterial populations were considered to be nitrate-nitrogen ($\text{NO}_3\text{-N}$) and dissolved organic nitrogen. Among the environmental parameters that commonly influence the property of microbial cells in nature, the concentration of essential nutrients is of particular importance. Natural ecosystems frequently are virtually depleted of one or more of these nutrients (Veldkamp & Jannasch, 1972) as a consequence of the (potentially vigorous) metabolic activities of indigenous microbial populations. Hence, microbial growth in natural environments is nearly always nutrient limited so that "nutrient insufficiency is the most common environmental extreme to which microorganisms are routinely exposed" (Tempest & Neijssel, 1981).

Bacteria have to adapt to nutrient impoverished conditions, especially those bacteria introduced into an "alien" environment. One possible source of nutrients is the dissolved organic products excreted from photosynthetic and metabolically active bacteria. The potential ecological significance of photosynthetically produced dissolved organic carbon in secondary production, and the pathway of its return to the particulate phase of the food web was emphasized by Pomeroy (1974). Investigators demonstrated that algae in culture and from natural waters release some of the carbon fixed during photosynthesis into the medium (Berman & Holm-Hansen, 1974; Hellebust, 1974). This release varied from less than 1% to greater than 4% of the total fixed carbon. As reviewed by Hellebust (1974), a variety of compounds from vitamins to simple sugars were reported to be released; both dissolved inorganic carbon and O_2 concentrations, physiological state of the cells, temperature, and salinity affect the rate of dissolved organic carbon release.

The measurement of the relative amounts of dissolved organic carbon released by phytoplankton was expressed as the percentage of the total fixed carbon released. Generally, release was assumed to take place by passive diffusion through the cell plasmalemma, although evidence for active excretion was reported for some algae

under specific conditions (Hellebust, 1974). It was shown that a variety of "stress" conditions such as high light intensity (Hellebust, 1965) or low dissolved inorganic carbon concentrations (Tolbert, 1974) affect the percentage of fixed carbon released by algal cells. Smith and Wiebe (1976) showed that the rate of dissolved organic carbon release from both plankton samples and algal cultures was constant over a wide range of dissolved organic carbon concentrations and occurred independently of particulate organic carbon production. Bacteria initially presented with adequate nutrients to support growth and thereafter exposed to environments with low nutrient concentrations appear to adapt to the environmental challenge by utilizing one or more mechanisms for survival (Morita, 1985). It has been shown that populations of bacteria undergo a dramatic decrease in plate count but remain viable when analyzed by the direct viable count procedure. Bacteria which demonstrate this phenomenon, i.e., a viable but nonculturable state, include those in the genera *Escherichia*, *Vibrio*, *Shigella* (Colwell *et al.*, 1985; Xu *et al.*, 1982) and *Aeromonas* (Allen-Austin, 1984). These genera include representatives of both autochthonous and allochthonous aquatic microflora.

Many methods have been employed to isolate and study viable but nonculturable cells. Radiolabelling techniques have been used, both alone and in combination with other methods such as fluorescent labelling, to estimate cellular metabolic activity (Tabar & Neihof, 1982; Meyer-Reil, 1978). Autoradiograph has been combined with other methods such as immunofluorescence and are useful for distinguishing specific cells (Tabar & Neihof, 1982; Meyer-Reil, 1978). One method, the acridine orange direct count is widely adopted and generally accepted as being reliable for direct enumeration of total bacterial populations (Hobbie *et al.*, 1977).

Using the acridine orange direct counting method, it is possible to distinguish between living and inactive cells (Zimmerman *et al.*, 1978), based on the knowledge that acridine orange intercalates with nucleic acids and fluoresces red-orange in association with RNA and green in association with DNA (Hobbie *et al.*, 1977). A higher RNA/DNA ratio indicates active metabolism, whereas a low ratio indicates

metabolic inactivity, correlating red fluorescence with active cells and green fluorescence with inactive cells may therefore be valid.

It is possible to isolate small bacterial cells from marine environments that respond to addition of nutrients by increasing their size and undergoing cell division. Starvation of these large cells leads to size reduction (dwarfing). This behaviour is typical of copiotrophic bacteria and appears to be a response that allows the organism to withstand the stress of nutrient deficiency for long periods of time (Dawson *et al.*, 1981). One of the most significant changes that occurs in dwarf cells during starvation is the appearance of a number of proteins that are not present in growing cells (Amy & Morita, 1983b), but which may be important in the process of starvation-survival. The ability of starved bacteria to synthesize new proteins and respond to changes in the environment suggest that dwarf bacteria are metabolically active, a view that is reinforced by the demonstration that they contain high concentrations of ATP and other nucleoside triphosphate and maintain a sizable membrane potential at least during short-term starvation (Smigielski *et al.*, 1989).

3.2 Results

3.2.1 The effects of temperature and filtration on *A. hydrophila* survival in lake water microcosms

A. hydrophila grown in nutrient broth was inoculated into lake water microcosms and incubated at 4°, 15°, 25°, 30° and 37°C for up to 160 d. There were appreciable differences in *A. hydrophila* survival at these temperatures in untreated water which contained the natural microflora (Fig 3.1A) with the higher temperature resulting in the fastest decline in viable count. In Whatman-filtered water from which most protozoa and particulate matter were removed, the survival times were to some extent increased (Fig 3.1B) compared to those of untreated water (most notably at 15°C). Filtered-autoclaved water, from which viral particles i.e., bacteriophage and bacterial competitors were completely removed, showed the greatest increase in *A. hydrophila* survival at all temperatures (Fig 3.1C). T_{00} values (the time taken for a one log drop in viable count from the original inoculum size) and T_{99} values (the time for a two log drop) for these experiments are shown in Table 3.1.

3.2.2 The effect of cycloheximide on *A. hydrophila* survival in untreated lake water microcosms

Cycloheximide was used to eliminate eukaryotes present in untreated lake water microcosms. The addition of cycloheximide at a final concentration of 25 µg/ml to untreated lake water microcosms at 30°C (Fig 3.2A) or 15°C (Fig 3.2B) led to a slight decrease in the survival time of *A. hydrophila*. However cycloheximide at 50 or 100 µg/ml led to an increased survival time. T_{90} values for *A. hydrophila* survival are shown in Table 3.2. The long term survival characteristics of *A. hydrophila* were not altered by the addition of

Fig 3.1 The effect of temperature and filtration on *A.hydrophila* survival

A.hydrophila was grown in nutrient broth at 30°C then transferred to lake water microcosms. These were incubated in the dark at different temperatures.

- A. Untreated lake water
- B. Whatman-filtered lake water
- C. Filtered-autoclaved lake water

Log viable count represents colony forming unit per ml (cfu ml⁻¹).

Fig 3.1

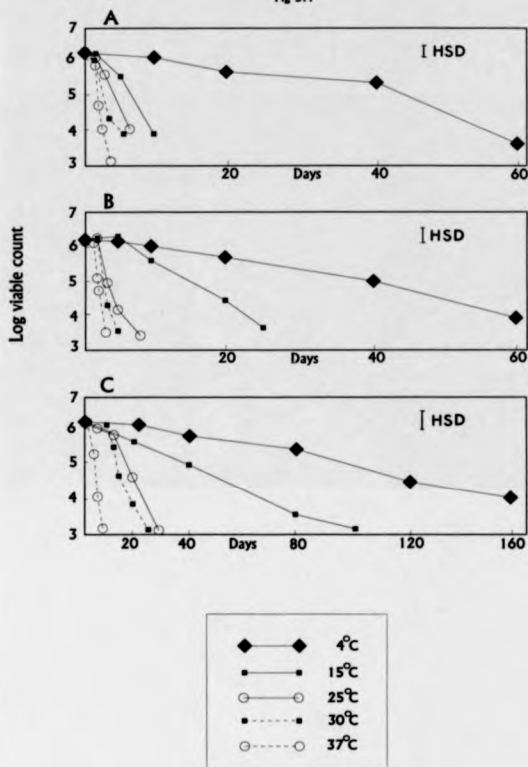


Fig 3.2 The effect of cycloheximide on *A.hydrophila* survival in untreated lake water microcosms

A.hydrophila was grown in nutrient broth at 30°C then transferred to untreated lake water microcosms. Cycloheximide was added to the microcosms which were then incubated in the dark at 30° or 15°C.

A. 30°C

B. 15°C

Log viable count represents cfu ml⁻¹.

Fig 3.2

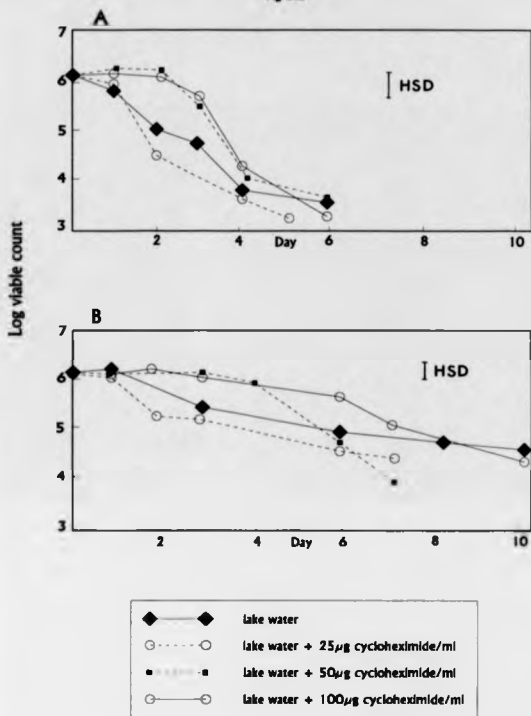


Table 3.1 T_{90} and T_{99} values for survival of *A.hydrophila* in lake water microcosms

Water sample	Temperature				Days
	37 °C	30 °C	25 °C	15 °C	4 °C
Untreated	1.5 (2.1)	2.3 (3.9)	2.7 (4.0)	5.1 (6.8)	29 (49)
Whatman filtered	2.0 (2.5)	2.7 (4.0)	3.3 (4.7)	14.4 (19.5)	34 (51.1)
Filtered autoclaved	2.2 (3.0)	15 (19.6)	17 (22.9)	23.8 (40)	92.9 (136.9)

Key

T_{90} (T_{99}) ; The time taken for a 1 log drop (2 log drop) in numbers from initial viable count.

Table 3.2 The effect of cycloheximide on T_{90} values of *A.hydrophila* in untreated lake water microcosms

Temperature	Cycloheximide ($\mu\text{g/ml}$)				Days
	0	25	50	100	
30 °C	1.7	1.3	3.2	3.3	
15 °C	2.7	1.9	5.1	6.5	

Key

T_{90} ; The time taken for a 1 log drop in numbers from initial viable count.

cycloheximide. After 6 d, survival in all the microcosms was virtually identical at 30°C and 15°C.

3.2.3 The effect of synthetic sewage on *A. hydrophila* survival in lake water microcosms

In order to study the effects of the addition of a complex of nutrients on survival, various concentrations of synthetic sewage were used to supplement filtered-autoclaved lake water microcosms at 30°C and 15°C. Synthetic sewage at a final concentration from 1 to 25% (v/v) caused an increase in cell numbers of *A. hydrophila* in lake water at both 30°C (Fig 3.3A) and 15°C (Fig 3.3B). The increase, up to 2 logs in 1 d was proportional to the synthetic sewage concentration added. This increase was followed by a slow decline in numbers at 30°C, but little or no decline at 15°C. An overall increase in numbers within 1 d was the same at 30°C and at 15°C.

3.2.4 The effect of carbon sources on *A. hydrophila* survival in lake water microcosms

The addition of glucose, succinate, glycerol, lactose and acetate at 5 mg-C or 500 mg-C/l to untreated lake water microcosms at 15°C led to an increase in survival times as well as an increase in cell population of *A. hydrophila*. There was notable growth on glucose at 5 mg-C/l, however this was followed by a rapid decline in cell numbers. In filtered-autoclaved water microcosms, all carbon sources tested led to an increase in viable counts with the largest effect being with glucose at both concentrations (Table 3.3A,B).

Fig 3.3 The effect of synthetic sewage on *A. hydrophila* survival in lake water microcosms

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. Synthetic sewage was added to microcosms which were then incubated in the dark at 30° or 15°C.

A. 30°C

B. 15°C

Log viable count represents cfu ml⁻¹.

Fig 3.3

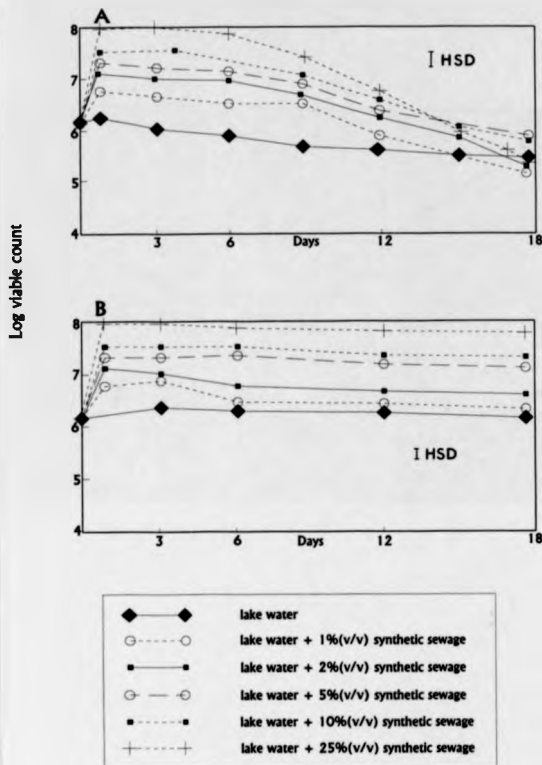


Table 3.3 The effect of carbon sources on *A. hydrophila* survival in lake water microcosms at 15°C

A. The effect of carbon sources at 5 mg-C/l on *A. hydrophila* survival

Water sample	Amendment	Log maximum population size/ml	T ₉₀ (d)
Untreated	None	6.35	5.4
	D-glucose	7.05	5.3
	Glycerol	6.74	8.0
	Lactose	6.95	7.3
	Succinate	6.73	6.9
	Acetate	6.74	7.9
Filtered - autoclaved	None	6.92	> 10
	D-glucose	7.29	> 10
	Glycerol	7.20	> 10
	Lactose	7.12	> 10
	Succinate	7.27	> 10
	Acetate	7.31	> 10

Key

Initial population size : 6.20 log cfu/ml

> 10 : Experiments terminated on day 10 before T₉₀ value was reached.

HSD in log population size; ± 0.09 (Untreated), ± 0.25 (Filtered autoclaved).

B. The effect of carbon sources at 500 mg-C/l on *A. hydrophila* survival

Water sample	Amendment	Log maximum population size/ml	T ₉₀ (d)
Untreated	None	6.36	5.4
	D-glucose	6.90	6.8
	Glycerol	6.89	8.1
	Lactose	6.90	7.7
	Succinate	6.75	6.7
	Acetate	6.75	9.5
Filtered - autoclaved	None	6.56	> 10
	D-glucose	7.20	> 10
	Glycerol	7.11	> 10
	Lactose	6.94	> 10
	Succinate	7.18	> 10
	Acetate	7.30	> 10

Key

Initial population size : 6.20 log cfu/ml

> 10 : Experiments terminated on day 10 before T₉₀ value was reached.

HSD in log population size; ± 0.23 (Untreated), ± 0.25 (Filtered autoclaved).

3.2.5 The effect of phosphate on *A.hydrophila* survival in lake water microcosms

The survival time of *A.hydrophila* was enhanced by the addition of phosphate at 50 and 100 mg PO₄/l to untreated water microcosms at 15°C but not with the addition of 10 mg PO₄/l. In filtered-autoclaved water microcosms phosphate at 50 or 100 mg/l led to an increase in viable counts for *A.hydrophila* (Table 3.4) but no increase in survival time.

3.2.6 The effect of nitrogen sources on *A.hydrophila* survival in lake water microcosms

3.2.6.1 The effect of different nitrogen sources on *A.hydrophila* survival at 15°C

Sodium nitrate and urea at 100 mg/l were used as nitrogen amendments. There was a little increase in survival times through the addition of sodium nitrate and urea to filtered-autoclaved lake water (Table 3.5A) and a small but reproducible increase in untreated lake water

3.2.6.2 The effect of different casein concentrations on *A.hydrophila* survival at 15°C

Various concentrations of casein from 0.1 to 100 mg/l were used for amendment. All concentrations casein tested led to an increase in *A.hydrophila* survival times probably proportionally to the concentration of casein used in Whatman-filtered water, there was also an increase in cell numbers in these microcosms with the largest increase in numbers with an addition of 100 mg/l.

Table 3.4 The effect of phosphate on *A. hydrophila* survival in lake water microcosms at 15°C

Water sample	Conc. (mg PO ₄ /l)	Log viable count/ml			T ₉₀ (d)
		Day 0	Day 2	Day 10	
Untreated	0	6.20	6.31	ND	5.4
	10	6.20	6.37	ND	5.8
	50	6.20	6.58	ND	9.3
	100	6.20	6.51	ND	9.5
Filtered - autoclaved	0	6.20	6.20	6.40	> 10
	10	6.20	6.18	6.37	> 10
	50	6.20	6.37	6.53	> 10
	100	6.20	6.75	6.83	> 10

Key

ND : Not determined

>10 : Experimenta terminated on day 10 before T₉₀ value was reached

HSD in log viable count : ± 0.17 (Untreated), ± 0.18 (Filtered autoclaved).

Table 3.5 The effect of nitrogen sources on *A. hydrophila* survival in lake water microcosms

A. The effect of urea, sodium nitrate and casein on population size and T_{90}

Amendment	Conc. (mg/l)	Log maximum population size/ml			T_{90} (d)		
		UNT	WF	FA	UNT	WF	FA
None	0	6.33	6.33	6.81	5.4	6.4	>10
Urea	100	6.46	ND	6.92	6.2	ND	>10
Sodium nitrate	100	6.59	ND	6.90	6.6	ND	>10
Casein	0.1	ND	6.33	6.22	ND	6.8	>10
"	1	ND	6.65	6.28	ND	8.9	>10
"	5	ND	6.47	6.30	ND	9.4	>10
"	10	ND	7.11	7.11	ND	>10	>10
"	50	ND	7.32	7.66	ND	>10	>10
"	100	ND	7.55	8.22	ND	>10	>10

Key Initial population size : 6.20 log cfu/ml
 UNT : Unfiltered lake water at 15°C
 WF : Whatman-filtered lake water 15°C
 FA : Filtered-autoclaved lake water 15°C
 >10 : Experiments terminated on day 10 before T_{90} value was reached.
 ND : Not determined
 HSD in log population size; ± 0.20 (UNT), ± 0.15 (WF), ± 0.24 (FA).

B. The effect of ammonium sulphate on population size and T_{90}

Conc. (mg-N/l)	Log maximum population size/ml				T_{90} (d)			
	UNT		FA		UNT		FA	
	15°C	30°C	15°C	30°C	15°C	30°C	15°C	30°C
0	5.75	4.88	6.36	6.21	2.7	1.8	>10	>10
0.1	6.22	4.88	6.39	6.35	4.5	1.8	>10	>10
1	6.15	4.92	6.36	6.39	3.8	1.9	>10	>10
10	6.21	5.30	6.61	6.36	3.9	2.3	>10	>10
20	6.24	5.36	6.45	6.29	9.7	2.6	>10	>10
50	6.26	6.23	6.54	6.41	7.5	3.1	>10	>10
100	6.45	6.21	6.47	6.56	7.5	3.0	>10	>10

Key Initial population size : 6.20 log cfu/ml
 UNT : Unfiltered lake water
 WF : Whatman-filtered lake water
 FA : Filtered-autoclaved lake water
 >10 : Experiments terminated on day 10 before T_{90} value was reached.
 HSD in log population size; ± 0.28 (UNT, 15°C), ± 0.28 (UNT, 30°C), ± 0.27 (FA, 15°C),
 ± 0.22 (FA, 30°C).

C. The effect of amino acid mixtures on population size and T_{90}

Amendment	Log maximum population size/ml		T_{90} (d)	
	UNT	FA	UNT	FA
None	6.32	6.92	5.4	>10
Glu / asp / leu	6.93	7.24	6.1	>10
Gly / ser / thr / his	6.86	7.40	10.4	>10
Ala / lys / aan	6.82	7.48	6.8	>10
Arg / trp / tyr	6.76	7.33	6.8	>10
Ile / pro / val	6.63	7.14	5.5	>10
Cys / met / phe	6.48	7.14	6.6	>10

Key Each amino acid added at 10 mg/l.
 Initial population size : 6.20 log cfu/ml
 UNT : Untreated lake water at 15°C
 FA : Filtered-autoclaved lake water at 15°C
 >10 : Experiments terminated on day 10 before T_{90} value was reached
 HSD in log population size: ± 0.19 (UNT), ± 0.12 (FA).

D. The effect of individual amino acids on population size and T_{90}

Amendment	Log maximum population size/ml	T_{90} (d)
None	6.67	2.4
L-serine	7.34	3.0-3.4
L-glutamine	7.34	2.7-4.0
L-arginine	6.95	3.1-3.6
Glycine	6.73	2.4-3.4
L-threonine	6.73	2.5-2.8
L-histidine	6.78	2.1-2.9

Amino acids were added at concentration from 10 to 500 mg/l to untreated lake water at 15°C.
 Maximum population size was always recorded in 500 mg/l amendment but growth occurred with all concentrations. HSD in log population size: ± 0.24 .

E. The effect of different concentration of L-serine and L-glutamine on population size and T_{90}

Concentration (mg/l)	L-serine		L-glutamine	
	Log maximum population size/ml	T_{90} (d)	Log maximum population size/ml	T_{90} (d)
0	6.27	6.4	6.20	6.4
0.1	6.25	7.9	6.20	9.5
1	6.23	6.8	6.25	8.5
2	6.61	8.8	6.36	8.3
5	6.67	9.3	6.33	8.1
10	6.70	>10	6.38	>10
50	6.81	>10	6.88	>10

All contained Whatman-filtered water and were incubated at 15°C.
 HSD in log population size: ± 0.15 (L-serine), ± 0.17 (L-glutamine).

There was also a concentration-dependent increase in viable counts with casein at all concentrations in filtered-autoclaved lake water, particularly a 2 log increase at 100 mg/l, the increase was followed by a slow decline from day 2 to day 10 (Table 3.5A).

3.2.6.3 The effect of different ammonium sulphate concentrations on *A. hydrophila* survival

Ammonium sulphate at 10 to 100 mg-N/l at 30°C and 0.1 to 100 mg-N/l at 15°C increased the survival time in untreated lake water microcosm. In particular ammonium sulphate at 20 to 100 mg-N/l at 15°C had a large effect on *A. hydrophila* survival. In filtered-autoclaved water at 30°C and 15°C all concentrations of ammonium sulphate tested led to a small but significant increase in the viable count of *A. hydrophila* (Table 3.5B) but in all cases the T_{90} value remained in excess of 10 d.

3.2.6.4 The effect of amino acid mixtures on *A. hydrophila* survival at 15°C

The addition of amino acid mixtures (final concentration of 10 mg/l each) as nitrogen amendments to untreated lake water microcosms resulted in an increase in the viable count as well as enhanced survival especially with a mixture of glycine, serine, threonine and histidine. In filtered-autoclaved lake water all amino acid mixtures led to an increase in the viable count of *A. hydrophila* (Table 3.5C).

3.2.6.5. The effect of individual amino acids on *A. hydrophila* survival at 15°C

In the above experiment *A. hydrophila* survival was enhanced notably in the presence of a mixture of glycine, serine, threonine and histidine. Therefore the

effects of these single amino acids were investigated in more detail. Serine, glutamine and arginine at 10 to 500 mg/l led to an increase in survival times as well as to an increase in the viable count in untreated lake water. However there was not a significant effect on *A. hydrophila* survival after the addition of glycine, threonine and histidine at the same concentration (Table 3.5D).

3.2.6.6. The effect of L-serine and L-glutamine on *A. hydrophila* survival at 15°C

As expected different concentrations of serine and glutamine added to Whatman-filtered lake water did cause an increase in survival times and in the viable count of *A. hydrophila*, with a concentration-dependent increase in survival and viable count with serine at 2 to 50 mg/l (Table 3.5E)

3.2.7 The effect of compounds released by microorganisms on *A. hydrophila* survival

3.2.7.1 The effect of compounds released by *Flavobacterium* on *A. hydrophila* survival

Various volumes of *Flavobacterium* were inoculated into dialysis bags in filtered-autoclaved lake water microcosms to examine the effect of products excreted by *Flavobacterium* on *A. hydrophila* survival under fluorescent light or in the dark at both 30° and 15°C. At 30°C primary products released by *Flavobacterium* inoculated at a volume of 0.1, 1, 5 and 10 ml led to an increase in survival times, and caused an increase in viable count of *A. hydrophila*, nearly concentration-dependently both in the light and in the dark. This was followed by a rapid decline in viable count in the light but a little decline in the dark.

Table 3.6 The effect of bacterial products on *A. hydrophila* survival

Bacteria in dialysis bag	Conc. (% v/v)	Log maximum population size/ml		T ₉₀ (d)	
		In the light	In the dark	In the light	In the dark
<i>Flavobacterium</i> , 30°C	0	6.58	6.33	9.1	>12
	0.1	7.22	6.75	10.1	>12
	1	7.18 ± 0.19 ^a	6.97 ± 0.13 ^a	9.9	>12
	5	7.27	7.0	9.6	>12
	10	7.35	7.40	>12	>12
<i>Flavobacterium</i> , 15°C	0	6.57	6.25	11.2	>12
	0.1	6.92	6.68	>12	>12
	1	7.04 ± 0.31 ^a	6.70 ± 0.16 ^a	>12	>12
	5	7.29	7.06	>12	>12
	10	7.36	7.15	>12	>12
<i>Anacystis</i> , 30°C	0	6.58	6.33	9.1	>12
	0.1	6.70	7.13	9.5	>12
	1	7.15 ± 0.22 ^a	7.07 ± 0.30 ^a	10.5	>12
	5	6.94	7.21	10.2	>12
	10	7.25	7.41	11.5	>12
<i>Anacystis</i> , 15°C	0	6.57	6.25	11.2	>12
	0.1	6.95	6.68	>12	>12
	1	7.0	6.79 ± 0.12 ^a	>12	>12
	5	7.04	7.06	>12	>12
	10	7.16	7.15	>12	>12

Key Initial population size of *A. hydrophila* : 6.20 log cfu/ml
 >12 : Experiments terminated on day 12 before T₉₀ value was reached
 a : HSD in log population size.

Table 3.7 The effect of algal products on *A. hydrophila* survival

Algae in dialysis bag	Conc. (% v/v)	Initial population size/ml		T ₉₀ (d)	
		In the light	In the dark	In the light	In the dark
<i>Chlorella</i> , 30°C	0	6.08	6.40	7.5	>12
	0.1	6.13	6.17	8.6	8.7
	1	7.02 ± 0.23 ^a	6.93 ± 0.24 ^a	8.3	9.8
	5	6.95	7.10	8.3	9.9
	10	7.03	7.15	9.5	>12
<i>Chlorella</i> , 15°C	0	6.57	6.25	11.2	>12
	0.1	6.90	6.47	>12	>12
	1	6.83 ± 0.13 ^a	6.60 ± 0.16 ^a	>12	>12
	5	6.97	6.85	>12	>12
	10	6.99	6.90	>12	>12
<i>Scenedesmus</i> , 30°C	0	6.08	6.40	7.5	>12
	0.1	6.45	6.80	9.8	10.4
	1	6.68 ± 0.16 ^a	6.76 ± 0.24 ^a	10.2	>12
	5	6.25	7.15	9.9	>12
	10	6.53	7.12	8.2	>12
<i>Scenedesmus</i> , 15°C	0	6.57	6.25	11.2	>12
	0.1	7.09	6.56	>12	>12
	1	7.31 ± 0.11 ^a	6.75 ± 0.23 ^a	>12	>12
	5	7.19	7.05	>12	>12
	10	7.21	6.98	>12	>12
<i>Chlamydomonas</i> , 30°C	0	6.08	6.40	7.5	>12
	0.1	6.48	6.30	>12	>12
	1	6.58 ± 0.12 ^a	6.35 ± 0.25 ^a	>12	>12
	5	6.69	6.63	>12	>12
	10	6.90	6.92	>12	>12
<i>Chlamydomonas</i> , 15°C	0	6.57	6.25	11.2	>12
	0.1	6.85	7.05	>12	>12
	1	6.96 ± 0.22 ^a	6.80 ± 0.30 ^a	>12	>12
	5	6.90	6.95	>12	>12
	10	6.97	7.13	>12	>12

Key Initial population size of *A. hydrophila* : 6.20 log cfu/ml
 >12 : Experiments terminated on day 12 before T₉₀ value was reached.
 a : HSD in log population size.

Similarly at 15°C there was a concentration-dependent increase in survival times as well as cell populations of *A.hydrophila* with slow decline of numbers in the light but no significant decline in the dark during incubation period. These changes must have been due to the compounds released from *Flavobacterium* in dialysis bags (Table 3.6).

3.2.7.2 The effect of compounds released by *Anacystis* on *A.hydrophila* survival

The products released from *Anacystis* in dialysis bags resulted in an increase in survival times and viable counts of *A.hydrophila* under fluorescent illumination and in the dark at 30°C. This was followed by a rapid decline in numbers in the illuminated samples but there was little decline in the samples incubated in the dark. At 15°C essentially similar results were observed as with the *Flavobacterium* inoculation into dialysis bags at 15°C. There was a concentration-dependent increase in survival times and population growth both in the light and in the dark without significant decline in numbers during the 12 d incubation period (Table 3.6).

3.2.7.3 The effect of compounds released by *Chlorella*, *Scenedesmus* and *Chlamydomonas* on *A.hydrophila* survival

In order to investigate the effect of algal products on *A.hydrophila* survival *Chlorella*, *Scenedesmus*, and *Chlamydomonas* were used. Both the viable counts and survival times of *A.hydrophila* were increased after the inoculation of these algae into dialysis bags at a volume of 0.1, 1, 5 and 10 mls respectively regardless of the incubation temperatures used or of incubation in the light or in the dark. There was a decline in cell numbers for *A.hydrophila* at 30°C but no significant decline at 15°C (Table 3.7).

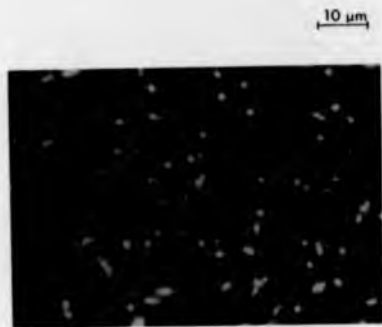


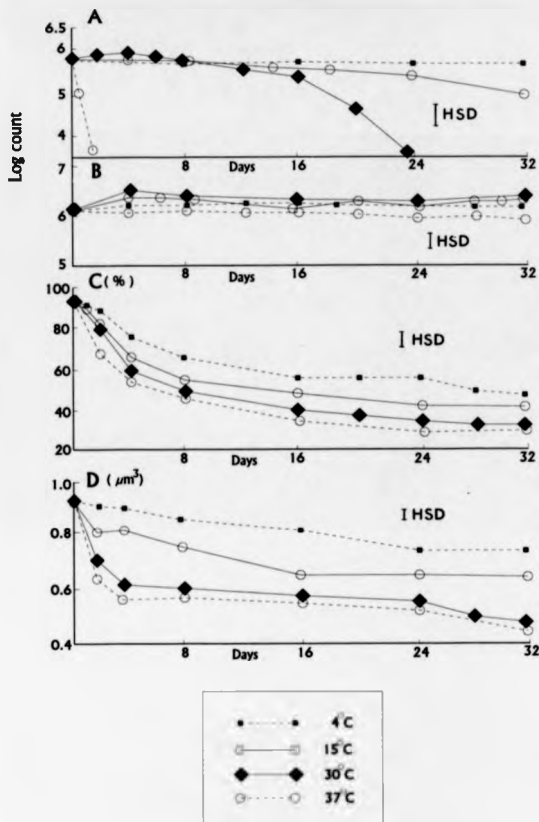
Fig 3.4 Respiring (red fluorescence) and nonrespiring (green fluorescence) cells, by the method of Zimmermann *et al.* (1978).

Fig 3.5 The effect of temperature on cell count, respiration and volume of *A. hydrophila*

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. These were incubated in the dark at different temperatures.

- A. Viable count (cfu ml⁻¹)
- B. Total count (ml⁻¹)
- C. Respiring cells
- D. Cell volume

Fig 3.5



3.2.8 The effect of temperature on cell count, respiration and volume of *A. hydrophila*

In this experiments, viable counts, total count, respiring cells and cell volume of *A. hydrophila* were investigated in filtered-autoclaved lake water microcosms at different temperatures. The viable count declined in proportion to temperature with T_{90} values of 0.8, 16.7, 31 and >32 d in the order of 37°, 30°, 15° and 4°C over the 32 d experimental period (Fig 3.5A). The total number of cells, as determined by direct counts, started slightly higher than the viable counts at the onset of the experiment and showed no significant variation during the time period at all temperatures used (Fig 3.5B). The fraction of the total population which was actively respiring during starvation at all temperatures tested, as determined by the INT method, is also shown in Fig 3.5C. At the onset of starvation, 97.2 % of the cells in the suspension were actively respiring as demonstrated by their deposition of INT-formazan granules, which is shown in Fig 3.4. During starvation the percentage of endogenously respiring cells decreased to 55, 48.8, 36.1, and 32.9% of the total count at 4°, 15°, 30° and 37°C respectively by day 32. Fig 3.5D shows the changes in cell volume of *A. hydrophila*. The starvation conditions caused the reduction of cell volume in the filtered-autoclaved lake water microcosms at all temperatures tested here. The reduction of cell volume was also dependent upon the temperature. The lower the temperature of incubation, the slower was the decrease of volume, with the reduction to 0.74, 0.65, 0.49, and 0.46 μm^3 from initial cell volume (0.92 μm^3) at temperatures of 4°, 15°, 30°, and 37°C respectively after 32-d starvation.

3.2.9 The effect of nutrients on the cell volume of *A. hydrophila*

The addition of carbon sources, such as D-glucose, succinate, glycerol, lactose and acetate, resulted in a delayed effect on the reduction of the cell volume of *A. hydrophila* in filtered-autoclaved lake water microcosms at 30°C. After the addition of carbon sources the cell volume decreased by between 0.1 and 0.27 μm^3 compared to 0.32 μm^3 in the control within 4 d. A slow decrease in cell volumes was detected for the next 32 d in all samples (Fig 3.6A). Similar results were obtained by the addition of phosphate or nitrogen sources such as casein, glutamine and serine to lake water. The decrease in cell volume of *A. hydrophila* after the addition of phosphate or nitrogen sources was 0.2 to 0.28 μm^3 drop compared with 0.32 μm^3 drop in the control with the first 4 d (Fig 3.6B). After the addition of a nutrient source, the decrease in cell volume on a 40d starvation period was always less than in the control.

3.2.10 The effect of sodium chloride on cell count and respiration of *A. hydrophila*

Different concentrations of sodium chloride were added to filtered-autoclaved lake water microcosms to investigate the effects of sodium chloride on viable count, total count and the number of respiring cells at 30°C. The total count remained constant during the 28 d starvation period, the viable count however declined gradually at 0 to 3% sodium chloride, and very rapidly at 5 or 7% sodium chloride (Fig 3.7A, 3.7B). When *A. hydrophila* was subject to osmotic shock, the percentage of respiring cells declined gradually at 0.5% sodium chloride by a drop of 28% and more rapidly at 1.5 to 7% sodium chloride with a drop of 58.5 to 63% compared to a drop of 18.1% in the control within the first 6 days. A slow decline was then maintained for the rest of the starvation period.

Fig 3.6 The effect of nutrients on cell volume of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. Nutrient sources were added to the flasks and these were incubated in the dark at 30°C.

Fig 3.6

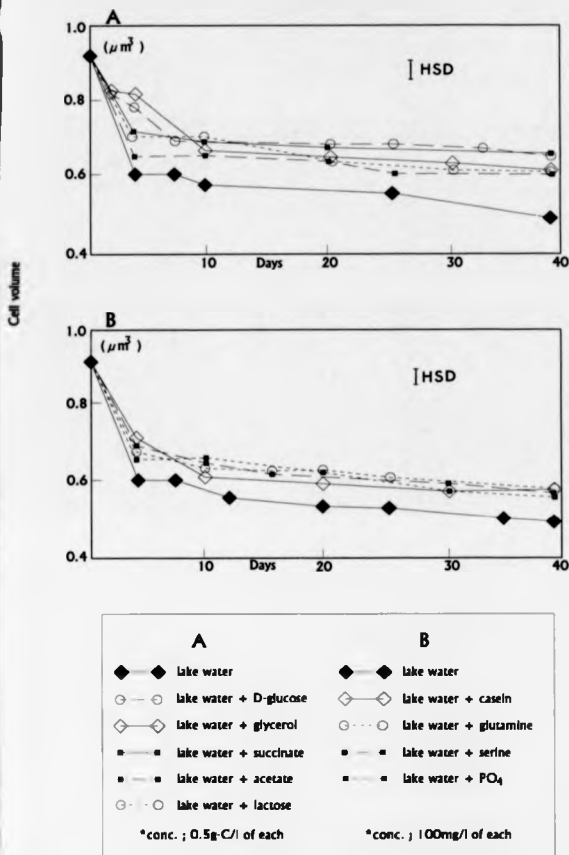
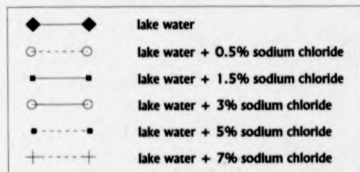
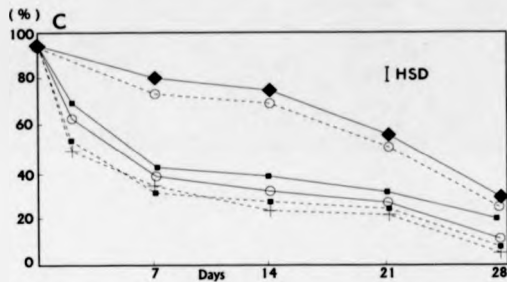
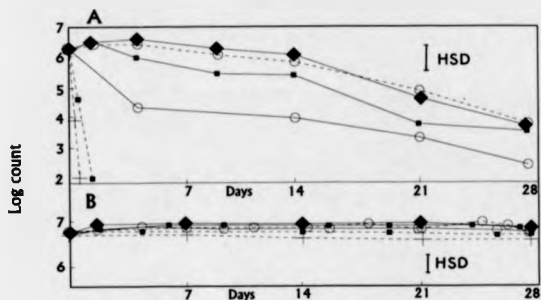


Fig 3.7 The effect of sodium chloride on cell count and respiration of *A. hydrophila*

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. All flasks were incubated in the dark at 30°C.

- A. Viable count (cfu ml⁻¹)
- B. Total count (ml⁻¹)
- C. Respiring cells

Fig 3.7



It is remarkable that although the viable count had decreased by 4-logs over the first 2 days of the starvation period (Fig 3.7A) after the addition of 5 % and 7 % sodium chloride, almost 50 % of the cells in both samples remained capable of respiration (Fig 3.7C). Even after 28 d incubation between 3 and 7 % of cells were still capable of respiration in these high sodium chloride concentrations.

3.2.11 The effect of osmoprotectants on the recovery of *A. hydrophila* from osmotic shock

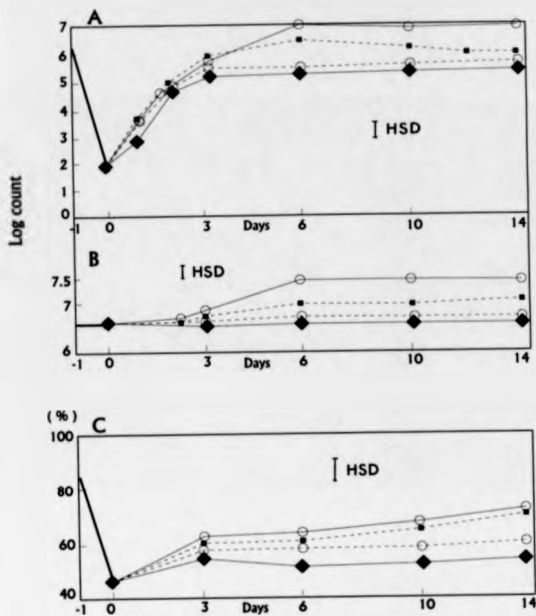
The viable count of *A. hydrophila* dropped to less than 0.01 % of the initial count in the presence of 5% sodium chloride at 30°C within the first 24 h. After the cells were removed from the sodium chloride by centrifugation the addition of betaine plus proline or betaine to filtered-autoclaved water at 15°C resulted in a more rapid recovery of cell numbers from osmotic shock, compared to the recovery in the control or in the presence of proline alone. The population recovered to its initial density after the removal of sodium chloride within 4.1 and 4.6 d in the presence of betaine plus proline and betaine respectively with no further increase in numbers from day 6 to 14. The viable count only increased to 10% of the initial count within approximately 4 days in the presence of proline alone and in the control with a further slow increase in numbers up to day 14 (Fig 3.8A). Total cell numbers did not change in the presence of 5% sodium chloride for 1 day although viable counts declined dramatically. After removing sodium chloride, the total cell count increased to 1.7×10^7 and 5.9×10^6 from 3.2×10^6 by day 5, with betaine plus proline and betaine respectively. No significant fluctuations over the time period were observed with proline alone and in the control over the 14 d starvation period (Fig 3.8B). The respiring cell percentage (84% at the onset of experiment) decreased to 46% in the presence of sodium chloride within 1 d but the

Fig 3.8 The effect of osmoprotectants on the recovery of *A. hydrophila* from osmotic shock

A. hydrophila was grown in nutrient broth at 30°C then transferred to a filtered-autoclaved lake water microcosm containing 5% sodium chloride and incubated in the dark at 30°C for 1 day. After centrifugation to pellet the cells, *A. hydrophila* was washed three times in filtered-autoclaved lake water and transferred to fresh filtered-autoclaved lake water microcosms containing osmoprotectants at 15°C.

- A. Viable count (cfu ml⁻¹)
- B. Total count (ml⁻¹)
- C. Respiring cells

Fig 3.8



percentage gradually recovered up to 78, 75 and 64% on betaine plus proline, betaine and proline respectively compared with 54% in the control (Fig 3.8C).

3.2.12 The effect of cadmium chloride on cell count and respiration of *A. hydrophila*

The addition of cadmium chloride to filtered-autoclaved water at 30°C caused a similar result as shown in Fig 3.7 for the effects of osmotic shock on *A. hydrophila* in lake water. The viable count of *A. hydrophila* declined in a concentration-dependent fashion, with a t_{90} value of between 0.3 and 1 d with 2 to 100 mg/l, and of 2.3 d for 1 mg/l cadmium chloride compared to 14.4 d for the control (Fig 3.9A). The total counts were nearly constant at all concentrations of cadmium chloride tested except the control showed an increase for the first 2 days followed by little variation during the rest of the 32 d starvation period (Fig 3.9B). The percentage of cells capable of respiring was 95% at zero time but this decreased to 53-63% in the presence of 1-100mg/l cadmium chloride within 2 days compared to a decrease to 80% for the control. Although the viable count of *A. hydrophila* on nutrient agar plates fell to less than 1% of the initial viable count in the presence of 2-100 mg/l cadmium chloride and below 10% in the presence of 1 mg/l cadmium chloride within 2 days, the percentage of the cells capable of respiration remained above 50% of the total population (Fig 3.9C).

3.2.13 The effect of nutrient deprivation on cell count and respiration of *A. hydrophila*

A. hydrophila was also subjected to starvation specifically for carbon or nitrogen sources, by incubation in minimal salts media rather than in lake water.

Fig 3.9 The effect of cadmium chloride on cell count and respiration of *A. hydrophila*

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms with different concentrations of cadmium chloride. These were incubated in the dark at 30°C.

- A. Viable count (cfu ml⁻¹)
- B. Total count (ml⁻¹)
- C. Respiring cells

Fig 3.9

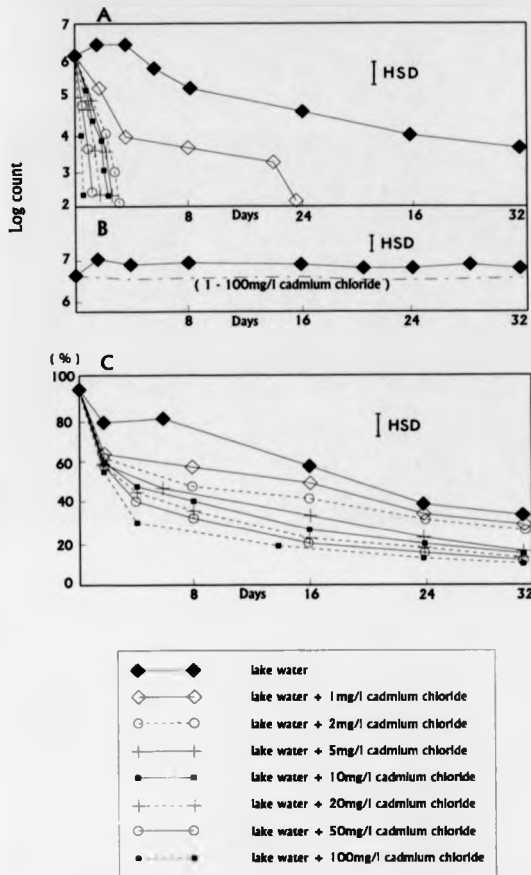
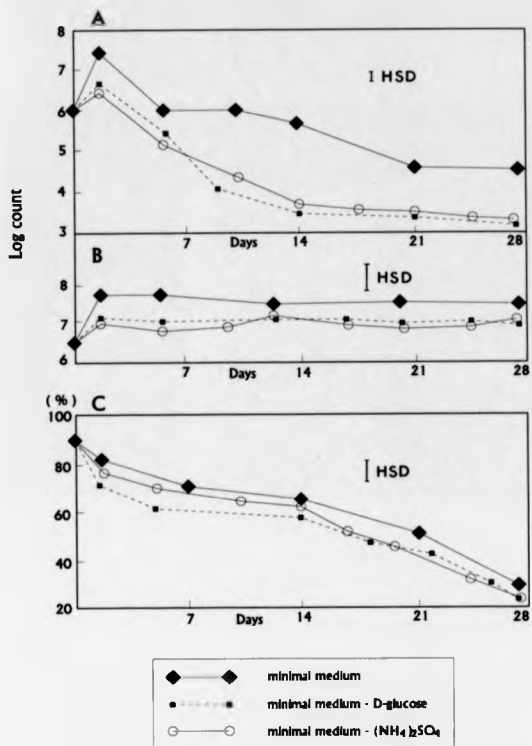


Fig 3.10 The effect of nutrient deprivation on cell count and respiration of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to minimal medium, minimal medium deprived of a carbon source or minimal medium deprived of a nitrogen source. All flasks were incubated in the dark at 30°C.

- A. Viable count(cfu ml⁻¹)
- B. Total count (ml⁻¹)
- C. Respiring cells

Fig 3.10



The viable counts declined more rapidly , with the t_{90} values of 6.6 and 6.0 d respectively for carbon and nitrogen, compared to 17.5 d in the control (Fig 3.10). Again there was little change in total count in minimal medium and in minimal medium minus the carbon or nitrogen source over the 28 d starvation period, after an initial increase in the population for the first 2 d (Fig 3.10B). As expected the deprivation of nutrient sources such as carbon and nitrogen caused a reduction in the percentage of cells capable of respiring (Fig 3.10C). Again there was a greater number of cells able to respire than capable of growth as shown by the higher count of respiring cells to viable count on nutrient agar.

3.3 Discussion

In natural environments microorganisms are subject to fluxes of nutrients which may encompass conditions of surfeit, suboptimal amounts or the complete absence of substrates. It is vital, therefore, that they should be able to survive the periods of deprivation and recover rapidly when the supply of nutrients improves (Dawes, 1985). In aquatic systems, nutrients are usually absorbed until the concentration decreases to levels that are sufficient for the growth of only those organisms with good transport systems, or ones that can grow very slowly.

Factors involved in the survival of microorganisms under starvation conditions are complex and frequently interactive, although in the final analysis it is likely to be the exhaustion of an intracellular source of energy which results in death of the organism. Parameters that have been implicated in the survival of vegetative bacteria include the substrates available for endogenous metabolism, which is defined as the total metabolic reactions that occur when the bacterial cell is deprived of compounds or elements that may serve specifically as exogenous substrates (Dawes and Ribbons, 1962), the possession of storage compounds, the energy of maintenance requirement, the adenylate energy charge and the preservation of a membrane potential (Dawes, 1985).

The majority of bacteria are able to survive, often for prolonged periods, in the absence of nutrients - conditions of so-called "starvation survival" which is defined as a physiological state resulting from an insufficient level of nutrients, especially the lack of biological energy, to provide for growth and reproduction (Morita, 1988). Aquaeous suspensions of facultative bacteria displayed measurable respiratory quotients indicating the oxidation of intracellular material, presumably to furnish energy of maintenance and, possibly, permit the resynthesis from expendable endogenous carbon sources of essential cellular components that might be degraded during starvation (Dawes, 1985). As a

result of there being an insufficient amount of energy in the ecosystem, most microbes exist in some stage of starvation, depending upon when the cells have last been exposed to sufficient nutrients to permit growth and reproduction. When cells are faced with a lack of nutrients, four patterns of starvation survival result (Morita, 1985). These patterns are (1) the cells increase in number and then decline to a constant number, (2) the cell number remains constant (3) the cells increase in number and then remain constant, and (4) the cells decrease in number to a constant level.

In the study of Marden *et al.* (1985), it was suggested that the starvation-survival process is an immediate response probably due to energy deprivation and which might subsequently be induced by lack of nutrients, may induce an initial energy-dependent reorganization which involves both increased endogenous respiration and decreased rate of recovery, leads to rapid loss of polyhydroxybutyric acid, and results in production and release of outer membrane vesicles from some bacterial strains which is related to time of starvation and a decrease in cell volume.

Filtered-autoclaved water was routinely used in these experiments as a source of starvation medium. Others notably Morita have used a series of very artificial starvation media ranging from distilled water through buffers to growth media minus one key ingredient such as a carbon or nitrogen sources. It was felt that a more 'natural' starvation medium would equate to the environment that the cells would be exposed to under starvation conditions. It was realised that survival could be influenced by seasonal changes particularly as seasonal changes would alter the numbers of algae and other products in the water and hence alter the amount and types of carbon source present in the water. For this reason controls were always used and experiments repeated using the same batch of water. Filtered-autoclaved water was used rather than untreated water as this would eliminate any effects due to the different numbers and types of bacteria, algae and protozoa which would be present in the water at different times of the

season. The results therefore should be interpreted by comparison rather than treating each experiments as an entity in itself. Longer survival is always compared to the control which in some cases could be longer than in another experiment.

It may also be argued that autoclaving water changes it from being the "natural" sample argued above. However there is no other method for producing the sterile water needed for these experiments. Filtering through so called bacterial filters even with a pore size of 0.2 μm does not eliminate all bacteria and certainly does not eliminate bacteriophage (Flint, 1986; Lim & Flint, 1989). These can adversely affect any long term experiments although for short term experiments there effects are probably minimal (Flint, 1986). Autoclaving can alter the composition but this was felt to be a minor inconvenience alongside the major benefits of eliminating the bacterial and viral component of the water.

The results presented here, with the survival of *A. hydrophila* being compared in microcosms containing untreated lake water, Whatman-filtered lake water and filtered-autoclaved lake water at different temperatures suggest that the survival of *A. hydrophila* in lake water is dependent on both the absence of the natural microflora and the temperature of the water. The survival time of *A. hydrophila* was increased greatly in the order of filtered-autoclaved > Whatman-filtered > untreated water. Survival also relied on temperature with survival at 4° > 5° > 25° > 30° > 37°C for any lake water sample. Essentially the same results reported by Flint (1987), who suggested that temperature and competition for nutrients are the dominant factors most likely to affect the survival of *E. coli* in fresh water environments. In untreated water samples, cell numbers disappeared with t_{90} values between 1.5 and 29.0 d at temperatures from 4° to 37°C.

The relationship between survival times and temperature has been reported previously in other studies using *E. coli* in fresh or estuarine environments (McCambridge & McMeekin, 1981; Flint, 1987; Lim & Flint, 1989). Filtration

of lake water through Whatman No.1 filter paper which effectively removed most of the protozoa and the particulate material from the water sample, increased the survival times for *A.hydrophila* at each temperature. This would suggest that protozoa could be a factor in the disappearance of *A.hydrophila*, particularly at 15°C. At this temperature, the t_{90} value for *A.hydrophila* survival was increased by 4 days in the absence of protozoa. In other studies protozoan predation has been shown to be a significant factor in the fall in bacterial population size in lake water. Protozoan populations increase in size as the bacterial density falls, and the suppression of protozoa can lead to the elimination or delay in the decline of the bacterial population. The addition of protozoa to lake water in which indigenous protozoa have been suppressed will produce the same pattern of bacterial elimination as in untreated lake water (Scheuerman *et al.*, 1988; Gurijala & Alexander, 1990). A role for protozoa is evident by the increased survival of bacteria in lake water amended with eukaryotic inhibitors, a treatment that eliminated these predators.

Even when growth of bacteria was enhanced by the addition of high concentrations of nutrients, the impact of predation was noted since the addition of eukaryotic inhibitors to the lake water allowed for greater cell numbers (Alexander, 1981). The addition of cycloheximide to untreated lake water enhanced *A.hydrophila* survival but the increase at 15°C was less than that in Whatman-filtered water. It is possible that more than 100µg per ml of cycloheximide is needed to eliminate protozoa completely. Cycloheximide has been shown not to eliminate all the protozoa from a sample of eutrophic lake water (Sanders & Porter, 1986).

In the autoclaved water samples, the survival of *A.hydrophila* was greatly increased even at 30°C, the optimum growth temperature for *A.hydrophila*. The t_{90} value was up to 15 d in the autoclaved sample compared with 2.7 d or less in other samples. Autoclaving kills all competing predators (protozoa and bacteriophage) which are able to pass through Whatman-No.1 filter paper.

Scheuerman *et al.* (1988) suggested that bacterial competition induced a reduction in the response of *Pseudomonas* species to added nutrients in lake water. He suggested that introduced bacteria had to compete for the limited nutrient supply with the indigenous populations, so that only a portion of the supply of nutrients was available to the introduced population, once the supply was exhausted by the competitors, the introduced population would not multiply further. In addition Liang *et al.* (1982) observed that *Klebsiella pneumoniae* died rapidly in both sterile and non-sterile lake water, but no such rapid decline in numbers of *A. hydrophila* in sterile lake water was noted in the present study. The differences could result from the presence of toxins in the water in the earlier study, such toxins having been found in lake water samples by Klein and Alexander (1986).

LeChevallier and McFeters (1985) investigated interactions between *E. coli* and several common waterborne organisms and found the competition for limiting organic carbon to be responsible for the reduction of *E. coli* populations in drinking water. *Pseudomonas* and other native aquatic bacteria could easily compete with *E. coli* for available nutrients. Nutritionally deprived bacteria were probably more susceptible to secondary effects that may cause injury or cell death. Flint (1987) suggested that the effects of the natural microflora must be to compete with the *E. coli* for nutrients in the lake water rather than a direct toxic interaction. The presence of very small and very motile bacteria in river water samples filtered through a 0.45 μm millipore was examined microscopically by Flint (1987). These bacteria could not be cultured on nutrient agar incubated at temperatures from 10° to 30°C and consequently could not be identified although visual observation suggested that they resembled ultramicrobacteria. These were first defined by Torella and Morita (1981) as bacteria less than 0.3 μm in diameter in marine environments. It is probable that they could be nutrient starved or low nutrient-induced forms of heterotrophic, eutrophic, or autochthonous bacteria (Hood & MacDonell, 1987). It is also possible that

these could have been *Bdellovibrio* species but no attempts were made to identify them as such. In none of the experiment described here were *Bdellovibrio* identified.

Although no counts of bacteriophage were carried out here, previous studies of Flint (1984, 1987) indicated that the importance of bacteriophage in the decline in *E.coli* numbers in fresh water was only of importance after long term incubation at 15°C. Several investigators have reported that the high concentrations of bacteriophages in aquatic environments suggest that they may play important roles as agents of bacterial mortality and also mediate the genetic transfer between bacteria in nature. It has been suggested that the rate at which bacteriophage infection and proliferation occur, or have the capability of occurring, becomes important for analysis of the role of viruses in microbial ecology (Bergh *et al.*, 1989; Borsheim *et al.*, 1990). Flint (1987) suggested that, as a consequence, biological component of the water is primarily responsible for the disappearance of *E.coli* from untreated and treated fresh water samples and that temperature is a secondary, although very important factor.

As several investigators have postulated that nutrient competition could be the primary factor in bacterial disappearance in aquatic environments (LeChevallier and McFeters, 1985; Flint, 1987; Scheuerman *et al.*, 1988; Lim & Flint, 1989), the effects of various nutrient amendments on the survival of *A. hydrophila* were studied. As expected the addition of a complex mixture of nutrients, in the form of synthetic sewage, to filtered-autoclaved lake water gave an increase in the population number in proportion to the concentration of synthetic sewage added. Monfort & Baleux (1991) found that in the presence of highly concentrated effluent of sewage *Aeromonas* sp. increased not only in survival times but also in population size in brackish water. Schubert (1967) found that sewage pollution contributed to an increase in the number of aeromonads in natural waters although the aeromonads themselves were not considered to be of faecal origin. More *A. hydrophila* were found in domestic

water containing raw sewage compared to the water with no municipal wastes (Poffe and Op de Beeck, 1991). Lim and Flint (1989) also showed the survival time of *E.coli* to be increased in proportion to the concentration of synthetic sewage added, which indicated the importance of nutrients for the growth and survival of bacteria in aquatic environments.

The addition of readily utilizable carbon sources, such as glucose, glycerol, lactose, succinate and acetate to untreated and filtered-autoclaved water lead to an increase in the cell numbers as well as survival times of *A.hydrophila*, indicating that the cells might be carbon-limited under these starvation conditions and could compete with the natural microflora for carbon sources successfully in lake water. Van der Kooij and Hijnen (1988) suggested that mixtures of 18 carbohydrates and 18 carboxylic acids clearly enhanced the growth of *A.hydrophila* at individual compound concentrations above 1 μg of carbon per litre in drinking water. Van der Kooij and coworkers (1980) also reported that *A.hydrophila* could grow in tap water with glucose concentrations as low as 2.5 $\mu\text{g-C}$ per litre. The determined K_s value, 12 μg of glucose-C per litre, for *A.hydrophila* was very low as compared with some growth and transport constants of other bacteria on this substrate, e.g., *Pseudomonas aeruginosa*, 0.5 to 0.8 mg of glucose-C per litre (Guymon & Eagon, 1974); *P.fluorescens*, 72 μg of glucose-C per litre (Lynch & Franklin, 1978); *E.coli*, 72 μg of glucose-C per litre (Von Meyerburg, 1971). This suggests that aeromonads may compete effectively with these bacteria if glucose were to be growth limiting. On glucose at 10 μg of C per litre, *A.hydrophila* was found to grow rapidly with a lag phase less than 24 h, suggesting that its glucose uptake is controlled by a constitutive enzyme system with a high substrate affinity. An *A.hydrophila* isolate did not grow with acetate and succinate, with an observed lag period of 2 days or more, when these compounds were added at 1 mg of C per litre (Van der Kooij *et al.*, 1980).

The addition of orthophosphate at 50 or 100 mg per litre to untreated and filtered-autoclaved water led to an increase in numbers and survival times. It is likely that *A. hydrophila*, an indigenous aquatic bacterium, is phosphate-limited in lake water, and can utilize and compete for available phosphate successfully for its growth and survival even in the presence of other aquatic competitors. Rippey & Cabelli (1985) demonstrated that the rate of growth of aeromonads is increased proportionally by raising the level of nutrients, particularly phosphate, in natural waters. In the study of the survival of coliform bacteria, Lim & Flint (1989) showed no increase in viable counts and survival times of *E. coli* in lake water amended with orthophosphate. This suggests that although the lake water may have been phosphate-limited, either the *E. coli* grown in nutrient broth have sufficient intracellular reserves of phosphate to meet their requirements under starvation conditions or, although phosphate is an important element in metabolism and cell structure, phosphate plays little role in the survival of *E. coli* in fresh water.

The addition of nitrogen amendments, especially casein and amino acid mixtures, had a notable effect on the survival of *A. hydrophila* in untreated, Whatman-filtered and filtered-autoclaved lake water sample. The effects of casein in Whatman-filtered and filtered-autoclaved water was concentration dependent with longer survival times and higher growth in numbers being recorded at 100 mg per litre than at lower concentrations. Similar results were obtained with ammonium sulphate, amino acid mixtures and some individual amino acids such as serine, glutamine, arginine, glycine, threonine and histidine. Both survival and growth of *A. hydrophila* were increased in untreated and Whatman-filtered water on these nitrogen sources. A positive relationship between the nitrogen concentration with both growth and survival was observed for ammonium sulphate in untreated water and with serine and glutamine in Whatman-filtered water. The increase in numbers in filtered-autoclaved water indicated that *A. hydrophila* could utilize ammonium sulphate and amino acids

even in a starvation medium at 15°C. In the study by Hazen & Esch (1983) using diffusion chambers, the survival of *A. hydrophila* in estuarine water was increased at the discharge point of pulp mill effluent, which contained significant amounts of nitrogen sources such as nitrate, nitrite and ammonia. Flint (1987) suggested that the longer survival times for *E. coli* in sewage-polluted river water was due to the higher nitrogen content of this water rather than to the presence of extra organic materials which could be used as carbon sources.

It was demonstrated that a mixture of 21 amino acids, when added to drinking water, promoted growth of *A. hydrophila* at individual compound concentrations as low as 0.1 µg of C per litre (Van der Kooij & Hijnen, 1988). The high substrate affinities of *A. hydrophila* for amino acids suggested that aeromonads could compete successfully for these compounds at substrate conditions below a few micrograms per litre. As shown by Kay & Gronlund (1971), amino acids were regarded as suitable substrates which could be transported rapidly even at very low concentrations in the natural environment by the efficient uptake systems of *P. aeruginosa*. They also observed that transport of amino acids into cells of *P. aeruginosa* rapidly declined after a few minutes as a result of saturation of the pool with the unchanged compounds. Further uptake was dependent on incorporation of these amino acids from the pool into cellular proteins. The rate of C₄-acid transport into cells of *E. coli* also appeared to be determined by the rate at which these substrates were metabolized (Kay & Kornberg, 1969).

Burnison and Morita (1973) demonstrated competition for amino acid uptake in lake water. Despite high-affinity uptake systems for amino acids, pseudomonads appear to be unable to compete with many other bacteria resident in lake water, as may be concluded from the low percentage (usually < 1 % of the bacterial populations of surface and tap water) in which they are usually found in natural bacterial populations. Amako *et al.* (1987) showed that amino acids can influence the survival times of bacteria in lake water. They demonstrated

that *V.cholerae* survived longer in lake water supplemented with amino acids and suggested that competition for amino acids might be an important factor for the survival of bacteria in the aquatic environment. Under starvation conditions in phosphate buffer at 25°C, an unidentified marine Gram-negative rod and a marine *Pseudomonas* sp. survived better if there was an atmospheric nitrogen source available (Jones & Rhodes-Roberts, 1980; 1981). They put this increased survival down to the ability of these strains to scavenge for nitrogen under starvation conditions better than *E.coli* (Ks values 0.35 - 0.52 $\mu\text{M/l}$ for the marine bacteria compared with 1.75 $\mu\text{M/l}$ for *E.coli*).

In aquatic environments, ammonium ion and amino acids can be taken up by active transport coupled to protein synthesis (Kirchman & Hodson, 1984) or by passive diffusion (Brown & Johnson, 1977). The presence of energy-dependent uptake systems might facilitate scavenging for low levels of ammonium ion or amino acids, and these systems would be expected to be more efficient in natural isolates of oligotrophic environments than copiotrophs. Griffiths and Haight (1973) suggested that high affinity binding proteins were responsible for capture of amino acids in low nutrient aquatic environments. The ability to scavenge substrates when they are available only in very low concentrations apparently provides a selective advantage to those microorganisms demonstrating this characteristic. Marden *et al.* (1987) showed that leucine was rapidly taken up by starving cells of an unidentified Gram-negative marine bacterium and that uptake was followed by a period of increased protein synthesis and respiratory activity. Other studies noted that at the onset of starvation there is rapid turnover and degradation of intracellular macromolecules. Hood *et al.* (1986) found the decrease in levels of macromolecules i.e., lipids, carbohydrates, DNA, RNA and proteins, when *V.cholerae* was exposed to nutrient starvation. Amy *et al.* (1983a) also demonstrated, on a per cell basis, the rapid decline in cellular levels of nucleic acids and protein during the initial stage of starvation of a *Vibrio* species and they further suggested that cells undergo resynthesis of

macromolecules during recovery from starvation after nutrient addition (Amy *et al.*, 1983b).

Flint (1987) and Lim & Flint (1989) suggested that the occurrence of long-term starvation survival of *E. coli* in the absence of competitors in fresh waters was attributed to the slow shutdown of metabolism and was possibly dependent upon exogenous nitrogen source. The increase in survival times in the presence of amino acids may be a reflection of the availability of ammonium ions from the degradation of these compounds. Reeve *et al.* (1984) reviewed the role of protein degradation for survival of carbon-starved cells and found the survival time correlated with the rate of protein breakdown which could have made amino acids available or have released ammonium ions. The decreased protein content in an unidentified Gram-negative marine bacterium induced an increased free amino acid pool which could be used to satisfy the cells maintenance requirements (Marden *et al.*, 1987).

In experiments to investigate the effect of released compounds from microorganisms as nutrient sources on the survival of *A. hydrophila* it was shown that fluorescent light had a bactericidal effect on *A. hydrophila* survival in filtered-autoclaved lake water at both 30° and 15°C. Light, both the U.V and visible components of sunlight, is considered to be single important contributor to bacterial die-off in some natural waters (Eisenstark, 1971 ; Davies & Evison, 1991). The deleterious effect of fluorescent light on the survival of *A. hydrophila* appears to be derived from the low turbidity of the water which is greatly reduced in filtered-autoclaved water. It is likely that no or little turbidity allows light to penetrate through the water and this allows the damage to cells in spite of the low intensity of the fluorescent light. Davies and Evison (1991) showed that the absorbance of light by less coloured water such as treated, unchlorinated or marine water was much lower than that by untreated fresh water.

Primary products excreted by *Flavobacterium* led to an increase in *A. hydrophila* numbers both in the dark and in the fluorescent light, and reduced

the bactericidal effect in the presence of fluorescent light, prolonging cell viability. It might be considered that released compounds from *Flavobacterium* could reduce a lethal effect of fluorescent light by producing substances such as glycogen and polysaccharides which could absorb light and, in addition, serve as organic substrates for the growth and survival of *A. hydrophila*. It was similarly suggested that the capacity for greater survival of enteric bacteria in the presence of light might be due to the more favourable conditions which prevail in fresh water i.e., higher nutrient conditions and the presence of light-absorbing substances, such as humic and fulvic acids, which significantly reduce the loss of culturability in low salinity conditions (Davies & Evison, 1991).

The released compounds from *Anacystis* and algae contributed to an increase in numbers and survival of *A. hydrophila* both in fluorescent light and in the dark. These products appear to be dissolved organic carbon (DOC) which has been widely known to be released by cyanobacteria and algae in lake (Feuillade *et al.*, 1986), estuarine (Pregnull, 1983) and marine waters (Smith & Wiebe, 1976) during photosynthesis. This study showed that these products could be excreted in the dark as well as during photosynthesis under fluorescent illumination. Several physical, chemical and biological parameters were reported to affect the rate of DOC release by algae, such as light spectra and intensity, dissolved inorganic carbon, O₂ concentrations, physiological state of the cells, temperature and salinity (Smith & Wiebe, 1976). Microplanktonic algae in marine waters retained their darkness endurance capacity which is determined by their ecological origin (Antia, 1976). He suggested that algae could continue to tolerate darkness for at least 5-6 months under favourable conditions, such as optimum temperature. Konopka and Brock (1978) further showed that although the optimum temperature for photosynthesis of cyanobacteria was usually between 20° and 30°C, algal growth was not limited at temperatures below 15° and at 4°C where photosynthesis was usually 50 % of that at optimal temperatures.

Most of the DOC released by algae in natural waters during photosynthesis appeared to consist of variety of compounds from vitamins to simple sugars (Smith & Wiebe, 1976), relatively small labile molecules such as amino acids, organic acids and sugar phosphates (Pregnull, 1983) and glycolate (Krampitz & Yarris, 1983). Generally release has been assumed to take place by passive diffusion, although evidence for active excretion was reported for some algae under specific conditions (Hellebust, 1974). Smith and Wiebe (1976) postulated that the rate of DOC release from algal cultures is constant over a wide range of dissolved inorganic concentrations and occurs independently of particulate organic carbon production. As a result the intracellular DOC fated for release, exists either as a separate pool from that leading to particulate organic carbon production or that there is active metabolic control on one portion of a common pool. These released substances could be readily utilized through assimilation and respiration by the abundant heterotrophic microbes in their natural habitat (Pregnull, 1983). The soluble carbon excretion rate varied from less than 1% to greater than 40% (Smith & wiebe, 1976) and generally between 2% and 5% (Feuillade *et al.*, 1986) of photoassimilated carbon. Night excretion represented less than 10% of losses due to respiration, and the quantity of phytoplankton excretion products appear to be more than the quantity of organic compounds needed by heterotrophic organisms in the lake water (Feuillade *et al.*, 1986).

Krampitz and Yarris (1983) demonstrated that *Chlorella* excreted glycolate continuously in high yields. Tight coupling between the rates of carbon fixation and of absolute DOC excretion by *Chlorella* was observed over a wide range of irradiances with excretion ranging from 1 to 55 % of production. There was a direct correlation between temperature and DOC excretion in *Chlorella* and the percentage of excretion was increased under extreme irradiances and temperature when photosynthesis was depressed (Zlontnik & Dubnsky, 1989). Chromatographic analysis by Saks (1982) suggested that *Chlamydomonas* released only amino acids into the medium without any trace of carbohydrates or lipids.

Glycine and leucine were specifically identified as the only metabolites released to the medium in the nM range.

Viable and total counts, and respiring cell percentages of *A. hydrophila* were determined under various stress conditions such as starvation, osmotic shock, cadmium shock and nutrient deprivation. In all cases total cell numbers remained almost the same, while plate counts eventually decreased. This suggests no occurrence of cell lysis and the difference between viable counts and total counts could be derived from cell death or from the cells entering a dormant state under starvation conditions. Essentially the same results have been reported by Montfort and Baleux (1991) who found no significant changes in total counts of *Aeromonas* during 90 days in brackish water, as determined by acridine orange direct count, though no colonies were produced on nutrient agar. These cells may be either dead or viable but nonculturable, as has been described for *E. coli* and *V. cholerae* (Collwell *et al.*, 1985). Brettar and Hofle (1992) suggested that the discrepancy between plate counts and total counts could be explained by changes in the physiological state of the cells during exposure to low nutrient environments. Change of the uptake system for organic nutrients as well as loss of fitness due to starvation may be responsible for the loss of the ability to grow on agar plates.

In the experiments described in this chapter the percentage of cells capable of respiring gradually decreased temperature-dependently under starvation conditions, concentration dependently under osmotic or cadmium shock, and under carbon and nitrogen-deprived conditions. The respiring percentage, which rapidly dropped down in the presence of 5 % sodium chloride, recovered slowly after removing the sodium chloride from lake water or after the addition of osmoprotectants such as betaine and proline to lake water. At the same time the percentage of cells able to grow on agar plates also increased. The percentage of respiring cells was always greater than suggested by the viable counts on nutrient agar plates.

The difference between the viable and INT counts suggests the existence within the starving population of a subpopulation of nonviable but actively respiring cells which are more numerous than the viable cells. This phenomenon has been also observed in natural populations by autoradiography (Hoppe, 1976) and with a marine *Pseudomonas* species (Kurath & Morita, 1983). Amy *et al.* (1983a) measured 10 times more respiring cells compared with the viable cell count after 18 days of starvation. These data imply that many cells incapable of colony formation under the conditions used were able to respire sufficiently to deposit INT-formazan granules and showed an immediate drop of endogenous respiration rate upon starvation, as demonstrated by Nelson and Parkinson (1978) who reported rapid suppression of endogenous respiration during starvation-survival.

The ability to reduce the endogenous metabolic rate rapidly may be a primary survival requirement if bacteria are to endure during starvation conditions. Many investigations have suggested that reduced respiration and low endogenous metabolism are mechanisms enhancing survival under sparse nutrient conditions (Nelson & Parkinson, 1978 ; Novitsky & Morita, 1978 ; Kurath & Morita, 1983). In addition starved cells retained the ability to respire when challenged with substrate. The ability to respire was increased in cells starved for longer than 4 weeks when substrates were added to the medium (Amy *et al.*, 1983a). The maintenance of the ability to actively respire in spite of not being able to form a colony under starvation conditions indicates that cells probably enter a dormant stage. This has been referred to as a viable but nonculturable state. Dormancy is an important physiological adaptation contributing to the survival of bacteria suspended in the water column. Dormancy has been defined by Sussman and Halvorson (1966) as "any rest period or reversible interruption of the phenotypic development of an organism". They further divided dormancy into exogenous and constitutive dormancy. Exogenous dormancy is referred as "a condition in which development is delayed because of unfavourable

chemical or physical conditions of the environment". However most investigators are more familiar with constitutive dormancy as typified by endospores of the bacillaceae or myxospores of the myxobacteriaceae.

Many bacteria found in the aquatic environment are able to persist for significant periods of time as vegetative cells, exhausting energy reserves at a very slow rate via lowered metabolic activity (Novitsky & Morita, 1978). Byrd *et al.* (1991) showed viable but nonculturable cells of both Gram-negative and Gram-positive bacteria in starved drinking water. In a river water study Flint (1987) showed long-term survival of *E.coli* without any replication, suggesting that the cells must enter some type of dormant state. A study of *Klebsiella aerogenes* maintained under starvation conditions by Calcott and Postgate (1972), demonstrated that after 1 d about 20 % of the population was viable by culture methods but the remaining 80 % of the population was intact and responsive to mild changes in medium composition and concentration, suggesting the cells were viable but nonculturable. These results led to the formulation of the concept of a "pseudosenescent" state, in which bacteria lose the ability to multiply as a result of certain stresses but remain completely functional as individuals (Postgate, 1976). Stevenson (1978) expressed the belief that there exists certain stresses, such as starvation, that lead bacteria to decrease in size and activity and that once a particular stress has been removed normal development ensues.

The cell volume of *A. hydrophila* was influenced by temperature under starvation conditions and by exogenous nutrient sources. Cell volume decreased with increasing temperature under starvation conditions in lake water and the presence of substrates prevented the decrease in cell size. It was shown that bacteria in marine environments respond to nutrient deprivation by reducing their size. Hood *et al.* (1986) demonstrated the morphological changes of the bacteria upon starvation; the cells became coccoid and decreased in volume, losing over 90% of their original volume in 30 days. It was suggested that "size reduction" or "dwarfing" is a strategy for long term survival during periods

of nutrient stress (Dawson *et al.*, 1981). Kjelleberg & Hermansson (1984) reported that the progressive decrease in cell volume during starvation was due to the continuous degradation of exogenous material. Dwarfing leads to an increase in surface-to-volume ratio of the cell, which may aid bacteria in obtaining substrates from nutrient-poor environments (Morita, 1982). Moreover dwarf cells of *Vibrio* sp. were found to be more hydrophobic than growing cells, a characteristic that may aid in the adhesion of cells to solid-liquid interfaces (Kjelleberg & Hermansson, 1984).

It seems probable that size reduction is the visible manifestation of a cellular reorganization (or differentiation) that occurs in response to starvation, a reorganization that results in a metabolically quiescent form of bacterium that is nonetheless capable of responding rapidly to external stimuli such as the appearance of nutrients (Amy & Morita, 1983b). Wiebe *et al.* (1992) suggested similar results as shown in this experiment. Biovolume was affected by the temperature; volumes were smaller at higher temperatures and the largest cells were found at the lowest temperatures. Biovolume also showed a clear dependence on substrate concentration and quality; the largest cells were in the media with the highest nutrient content. Chrzanowski *et al.* (1988) suggested that a change in cell volume as a function of temperature is an intrinsic property of aquatic bacteria and is related to decreases in generation time. In estuarine and coastal waters, Palumbo *et al.* (1984) found cell volumes to be increased with increasing temperature, suggesting that the large phytoplankton blooms associated with the temperature increase may have increased nutrient availability.

Chapter 4

The Effect of Environmental Factors on Alkaline and Acid Phosphatase Activities of *Aeromonas hydrophila* in Lake Water Microcosms

4.1 Introduction

At least four transport systems are involved in the uptake of phosphate in *E. coli*. Of the four systems, two are primarily involved in the transport of inorganic phosphate (Pi) and two are involved in the transport of organo-phosphate metabolites. The two major routes of Pi uptake include a high affinity binding protein-dependent system (pst or phosphate-specific transport system) and low affinity membrane-bound system (pit) (Neidhardt *et al.*, 1987). Maintenance of the culturability of *E. coli* in sea water depended significantly on the presence of structures allowing access of phosphate ions to the periplasm, then to the cytoplasm of cells. Cells totally deprived of the two main phosphate transport systems (pit, pst) exhibited the highest loss of culturability. Most of this effect resulted from the loss of the high affinity pst system (Gauthier *et al.*, 1993).

Willsky *et al.* (1973) demonstrated, in more detail, two major Pi transport systems in *E. coli* K-12. The first system, designated the "pst system" was dependent upon the presence of the wild-type gene *PhoT* (ca. min 82 on the *E. coli* linkage map) for full activity. Another gene (*phoS*), closely linked to *phoT*, codes for the phosphate-binding protein, and forms an essential part of the complete transport process in the pst system. Each of these loci is involved in the regulation of alkaline phosphatase synthesis in *E. coli* (Eichols *et al.*, 1961). The involvement of a third gene, *pst*, in the pst system was also claimed for inorganic phosphate transport (Willsky *et al.*, 1973; Sprague *et al.*, 1975). The second system for phosphate transport in *E. coli* was designated the "pit system", for which one gene, *pit*, mapping at 62 min, was described (Sprague *et al.*, 1975).

Rosenberg *et al.* (1977) showed, using mutants of *E. coli* K-12, that one of the systems (pit) was fully constitutive, required no binding protein, and operated in spheroplasts. It permitted the complete exchange of intracellular phosphate with extracellular phosphate (or arsenate) and was completely inhibited by an uncoupler such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The high affinity

system was repressible by phosphate concentrations above 1 mM, required the phosphate-binding protein for full activity, and did not operate in spheroplasts. It catalyzed very little exchange between internal and external phosphate and was resistant to uncouplers. The maximal uptake velocities attained by the two systems were approximately the same, but the affinity for phosphate in the high affinity system was greater by two orders of magnitude. In strains in which both systems were fully operative the initial rate of uptake were nearly additive, and the systems appeared to interact with a common intracellular phosphate pool.

Alkaline phosphatase (EC 3.1.3.1) is a non-specific phosphomonoesterase that functions through formation of a covalent phosphoserine intermediate (E-P). The enzyme also catalyzes phosphoryl transfer reactions with various alcohols (Kim & Wyckoff, 1991). In *E. coli* the enzyme is involved in the acquisition of phosphate from esters when free inorganic phosphate is depleted. The *E. coli* enzyme has been studied most extensively by a variety of physicochemical techniques including nuclear magnetic resonance and electron spin resonance. Alkaline phosphatase of *E. coli* is a homodimer composed of two chemically identical subunits (Bradshaw *et al.*, 1981; Chang *et al.*, 1986) and is located in the periplasmic space. It is a metalloenzyme with two Zn^{2+} and one Mg^{2+} at each active site region. The important property of alkaline phosphatase is that it is enzymatically active only when it is exported across the cytoplasmic membrane into the periplasmic space (Michaelis *et al.*, 1986). If certain mutations are used to cause alkaline phosphatase to be retained in the cytoplasm, it is enzymatically inactive probably because the failure of the protein to assume a native, enzymatically active conformation in the cytoplasm results in its rapid degradation (Boyd *et al.*, 1987; Akiya *et al.*, 1989).

Derman and Beckwith (1991) have suggested that the inactivity of cytoplasmic alkaline phosphatase appears to be due, at least in part, to the absence of essential intrachain disulfide bonds. The cysteines, which are involved in disulfide bonds in the native alkaline phosphatase, were found to be fully reduced

when the protein was retained in the cytoplasm. Disulfide bond formation accompanied export and processing. The synthesis of alkaline phosphatase in *E. coli* is repressed by inorganic phosphate. Three loci are known to be involved in the synthesis of this enzyme: *phoA* - the structural gene and *phoR* and *phoS* - two regulatory loci. A mutation in *phoA* results in the inability to synthesize alkaline phosphatase and a mutation in either *phoR* or *phoS* results in constitutive synthesis of the enzyme. *PhoA* and *phoR* are linked and are located at position 10.5 on the genetic map; *phoS* is located remotely, at position 73.5. Three additional genetic markers - *lac*, *proC* and *tsx* are linked to the *phoA*, *phoR* region and the order of genes in this region of the *E. coli* chromosome, read in a clockwise direction was *lac*, *phoA*, *proC*, *phoR* and *tsx* (Bracha & Yagil, 1973).

Rogers and Reithel (1960) described two major phosphatases of *E. coli* active at an acid pH, but one of these was which extensively purified; this was a nucleoside 2'- or 3'-phosphatase, was virtually inactive against sugar phosphates. Most phosphate esters, including sugar monophosphates, phosphoryl ethanolamine, phosphoserine, 3' or 5' AMP, were hydrolyzed with an efficiency five to one hundred times lower than for para-nitrophenyl phosphate (PNPP). Similar conclusions were drawn by Hafkenschield (1968) who also observed that ATP and ADP were poor substrates compared to PNPP and concluded that the enzyme could not be an ATPase. Only fructose 1,6-diphosphate and 2,3-diphosphoglyceric acid were reported to be degraded with an efficiency approaching that of PNPP. Von Hofsten and Porath (1962) purified an acid phosphatase from extracts of *E. coli* and found it to be active against hexose phosphate monoesters although a complete summary of substrate specificity was not published. Neu and Heppel (1965) found that acid phosphatase was amongst the group of enzymes released into the medium when *E. coli* was converted into spheroplasts by treatment with lysozyme and EDTA, or when cells were subjected to osmotic shock.

An osmotic shock procedure caused the specific release from *E. coli* of a group of hydrolytic enzymes considered to be localized near the surface of the cell

(Dvorak *et al.*, 1967). They purified three distinct acid phosphatase fractions. one of these, accounting for about 70% of the activity against glucose 6-phosphate of cell extracts, was highly specific for hexose phosphates. The second fraction hydrolyzed a number of naturally occurring phosphate monoester but was most active against PNPP. The third one also hydrolysed PNPP. Acid phosphatase with a pH optimum of 2.5 (EC 3.1.3.2) is a monomeric protein and is relatively specific for molecules possessing numerous phosphoanhydride bonds, such as inorganic polyphosphates (Dassa & Bonquet, 1981). Most phosphomonoesters are not attacked by this enzyme, although an important exception is the synthetic substrate, PNPP. This acid phosphatase has been purified to homogeneity and culture conditions affecting its expression has been described (Dassa *et al.*, 1982). The synthesis of this phosphatase is repressed in exponentially growing bacteria but is induced as soon as cultures enter the stationary phase.

Four *E. coli* genes for acid phosphatase have been identified. (i) gene *Ush* specifies UDP glucose hydrolase and lies in the 11 min region of the chromosome (Beacham & Yagil, 1976); (ii) *appA*, the structural gene for an acid phosphoanhydride phosphohydrolase, which also cleaves PNPP, maps at min 25.5 (Dassa & Bonquet, 1985); (iii) gene *cpdB* encodes the 2'-3' cyclic phosphodiesterase, which is also active against PNPP and is located at min 96 (Beacham & Garrett, 1980) and (iiii) gene *agp*, which is the structural gene for an acid glucose-1-phosphatase (Pradel & Bonquet, 1988). These four genes have now been cloned, their restriction maps are known, and their nucleotide sequences have been partly or entirely elucidated. This chapter describes the effects of starvation and the amendment of a starvation medium on the induction of acid and alkaline phosphatase and on the activity of enzyme already induced in cells by growth in a phosphate-limited medium. The effects of other stresses upon the activity of phosphatase enzyme of *A. hydrophila* are also reported.

4.2 Results

4.2.1 Phosphatase activities of phosphate-limited *A. hydrophila*

A. hydrophila grown in a phosphate-limited medium was examined for phosphatase activities at a range of pHs from pH 4 to pH 11. Activity is significantly higher at alkaline pH with a peak in activity at or above pH 11 (Fig 4.1)

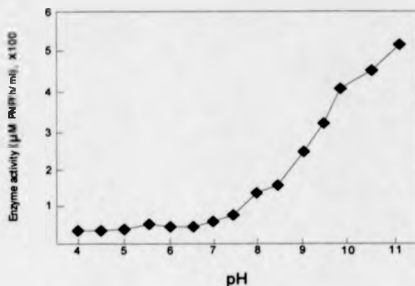
4.2.2 The effect of different temperatures on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Changes in the alkaline phosphatase activities of cells grown in a phosphate-limited medium (PLM) or a phosphate-rich medium (PRM) were examined in lake water at different temperatures. Alkaline phosphatase activity in PLM cells increased at all temperatures. At 37°C alkaline phosphatase activity slowly increased although there was a rapid decline in the viable count. However in PRM cells, no significant increase in alkaline phosphatase activity occurred at any of the temperatures used over a 36-day starvation period (Fig 4.2).

4.2.3 The effect of carbon sources on the alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Carbon sources, such as D-glucose, lactose, maltose, succinate and glycerol were added to lake water microcosms, the enzyme activities were measured in filtered-autoclaved lake water at 4°, 15°, 25°, 30° and 37°C. At 4°C, D-glucose, maltose, succinate and glycerol stimulated enzyme activities approximately 8.7 to 9.6 times for PLM cells and 6.6 to 100.7 times for PRM

Fig 4.1 pH profile of phosphatase activity in *A.hydrophila*.



Phosphatase activity was measured by pNPP hydrolysis at different pHs in 0.2 M TTA buffer. *A.hydrophila* was grown in phosphate-limited minimal medium at 30°C, harvested by centrifugation and resuspended in the buffer. Phosphatase activity was determined after incubation for 1 hour at 30°C.

Fig 4.2 The effect of temperature on alkaline phosphatase activity of *A.hydrophila* in lake water microcosms

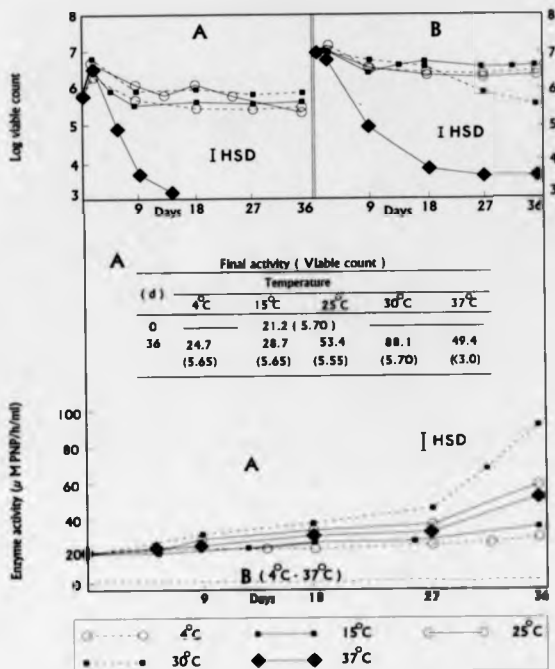
A.hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms. All flasks were incubated in the dark at different temperatures.

A. Phosphate-limited medium grown cells

B. Phosphate-rich medium grown cells

Log viable count represents cfu ml⁻¹.

Fig 4.2



cells in lake water over a 15 day starvation period, compared to 1.0 and 10.1 times respectively in the control but lactose had little or no effect on alkaline phosphatase activities in both PLM and PRM cells. There was little difference in the viable counts of the control and with added carbon sources except with D-glucose, succinate and lactose in PLM cells where a small increase in numbers was observed (Fig 4.3A, 4.3B). At the optimal growth temperature, 30°C, the enzyme activities of PLM cells rapidly increased in the presence of all the added carbon sources except lactose. Nearly maximum activities of enzyme were reached within 4 days with little variation afterward. Viable cell numbers also increased over the first 2 to 4 days. However apparently a very slow increase in enzymatic activity was exhibited with lactose although a large increase in viable count occurred within 8 d but eventually the activity increased up to 100 μM PNP/h/ml by day 28. In PRM cells the enzymatic activities were also greatly stimulated by carbon sources except lactose with which the activity increased slowly up to 92.3 μM PNP/h/ml on day 28. Here viable counts showed little or no increase (Fig 4.3C, 4.3D). Similar but less pronounced increases in activity were seen with PLM cells in lake water amended with carbon sources at 37°C although cell numbers declined to less than 1 % of the initial count during 15 d starvation period. With PRM cells enzymatic activities increased with added carbon sources for 2 d, then there was no significant variations during the starvation period (Fig 4.3E, 4.3F).

All carbon sources, except lactose, added to lake water at 15°C and 25°C displayed an apparently positive effect on the increase in the enzymatic activities in both PLM and PRM cells. A slightly quicker increase in the activities in PLM cells was found at 25°C compared with that at 15°C, reaching maximum activity within 6 d. The addition of lactose to lake water exerted a repression effect on the enzymatic activity at 15°C but slowly increased the activity at 25°C over a 15 d starvation period in PLM and PRM cells. At both temperatures

Fig 4.3 The effect of carbon sources on alkaline phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms. All carbon sources were added at a final concentration of 0.5 g-C/l. The flasks were incubated in the dark at different temperatures.

Phosphate-limited medium grown cells

- A. 4°C
- C. 30°C
- E. 37°C

Phosphate-rich medium grown cells

- B. 4°C
- D. 30°C
- F. 37°C

All carbon sources were added at 0.5g-C/l final concentration.

Log viable count represents cfu ml⁻¹.

Fig 4.3

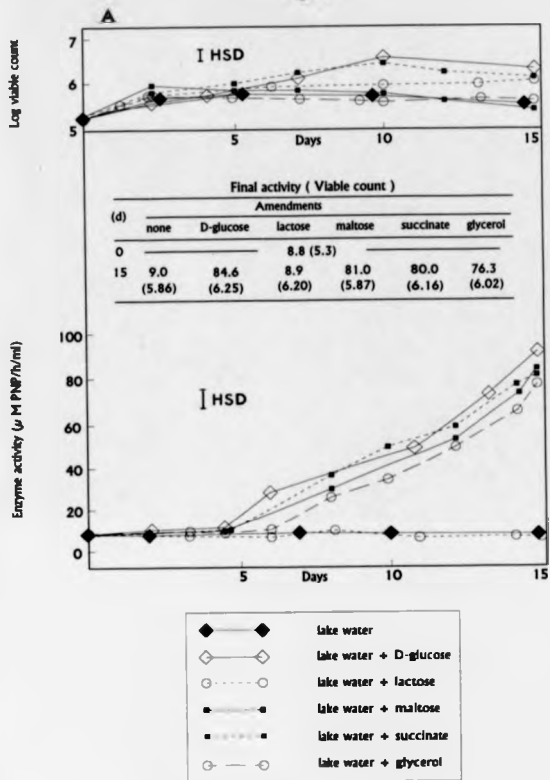


Fig 4.3

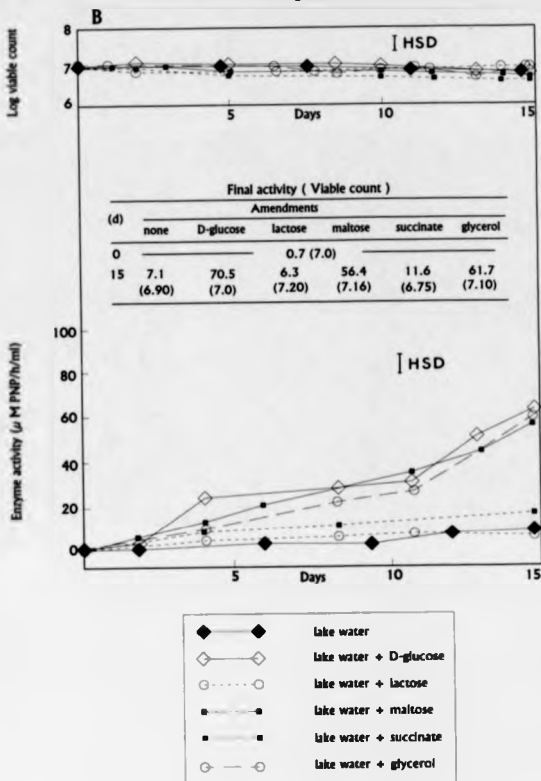


Fig 4.3

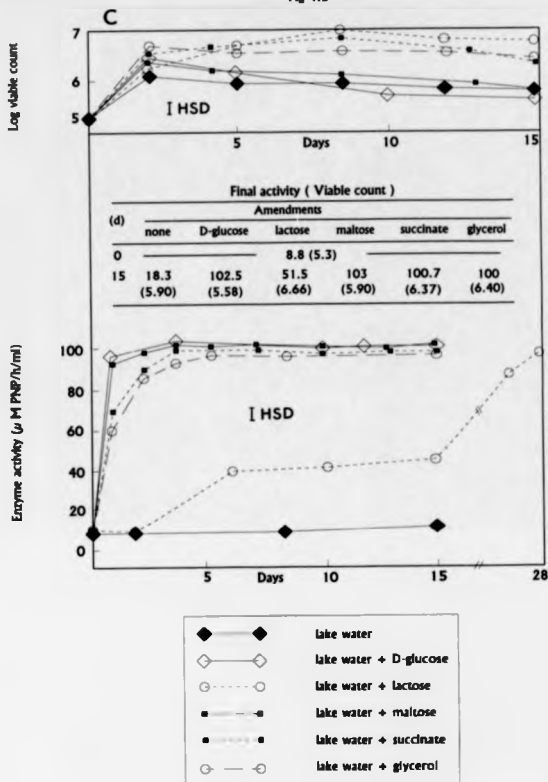


Fig 4.3

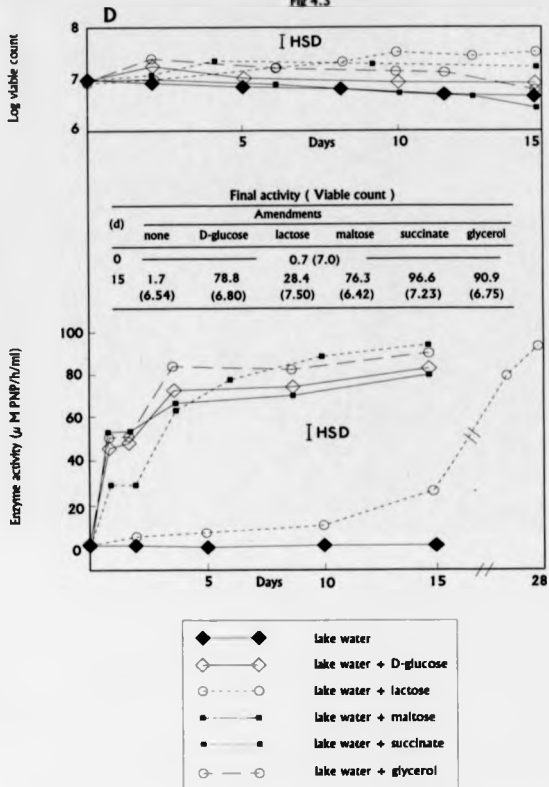


Fig 4.3

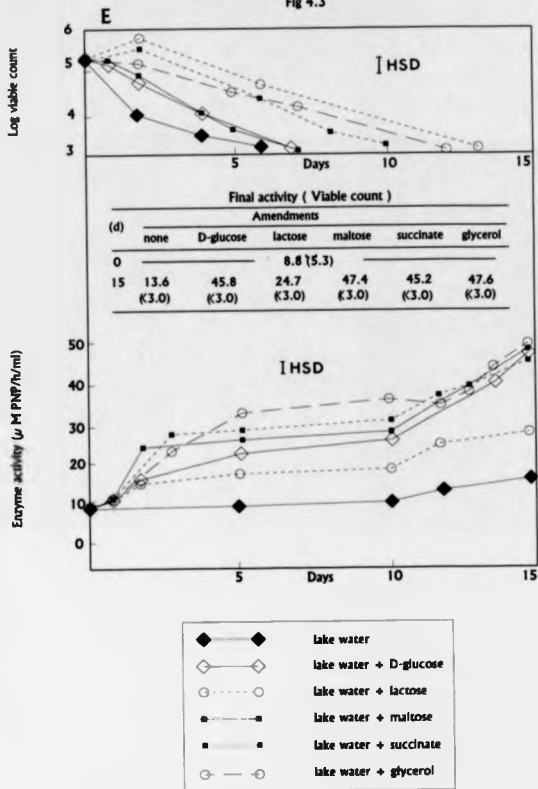


Fig 4.3

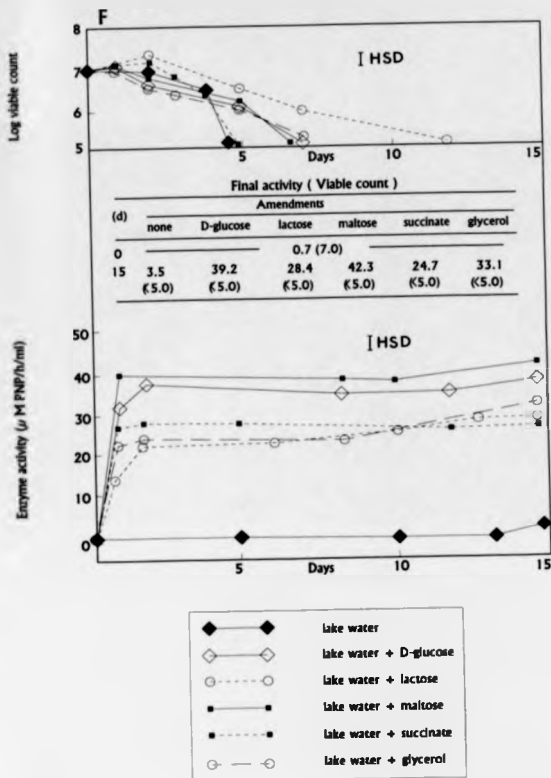


Table 4.1 The effect of carbon sources on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms at 15° and 25°C

Temp.	Amendment	Enzyme activity ($\mu\text{M PNP/ml/h}$)		Log viable count/ml	
		Day 2	Day 15	Day 2	Day 15
15°C (PL)	None	8.5	12.8	5.50	5.78
	D-glucose	81.0	100.0	6.80	5.94
	Glycerol	33.5	96.2	6.27	5.46
	Maltose	52.9	100.5	6.61	5.64
	Lactose	10.0	21.2	6.09	6.16
	Succinate	42.0	95.2	6.91	4.62
15°C (PR)	None	0.7	0.8	7.04	6.98
	D-glucose	67.7	98.7	7.20	7.11
	Glycerol	65.2	98.4	7.25	6.85
	Maltose	54.6	93.4	7.15	7.06
	Lactose	1.6	8.8	7.27	6.05
	Succinate	30.0	81.0	7.22	5.80
25°C (PL)	None	8.6	14.3	6.0	5.13
	D-glucose	96.9	100.5	6.21	5.30
	Glycerol	88.1	99.8	6.15	5.90
	Maltose	100.5	100.5	6.15	6.0
	Lactose	8.8	33.5	6.53	5.77
	Succinate	97.8	99.4	6.41	6.61
25°C (PR)	None	3.5	3.5	7.02	6.25
	D-glucose	89.0	101.2	7.37	6.50
	Glycerol	46.5	96.6	7.17	6.66
	Maltose	84.6	97.1	7.47	6.85
	Lactose	18.5	52.9	7.65	5.85
	Succinate	59.2	101.0	7.52	7.15

Key PL : Phosphate limited minimal medium grown cells
 PR : Phosphate rich medium grown cells
 Initial population size PL : $5.30 \log \text{ cfu/ml}$
 PR : $7.0 \log \text{ cfu/ml}$
 Initial enzyme activity PL : $8.8 \mu\text{M PNP/ml/h}$
 PR : $0.7 \mu\text{M PNP/ml/h}$

HSD

Temp.	HSD in Enzyme activity	HSD in Log viable count
15° C, PL	± 5.6	± 0.31
15° C, PR	± 4.3	± 0.14
25° C, PL	± 8.2	± 0.21
25° C, PR	± 3.9	± 0.09

the viable counts increased during the first 48 h starvation then declined more or less over the rest of the starvation period (Table 4.1).

4.2.4 The effect of nitrogen sources on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Several nitrogen sources, such as casein, sodium nitrate and ammonium sulphate, were used at a final concentration of 50 mg/l each to investigate their effects on the activity of alkaline phosphatase in filtered-autoclaved lake water. The activities in PLM cells continued to be stimulated up to 3.5 times with casein compared to 1.7 times in the control over a 20 d starvation period. There was an initial increase of at least 1 log in viable counts, followed by a slow decline after 2 days. Sodium nitrate and ammonium sulphate had a little effect on alkaline phosphatase activities although viable cell numbers changed as for the casein amended microcosm. In PRM cells, enzymatic activity was also, to a degree, increased with casein which also increased the survival times for *A. hydrophila* over the control in amended lake water. However there was no effect on enzymatic activity after the addition of the other two substrates (Fig 4.4A, 4.4B). Amino acids, such as leucine, glutamine, glycine, serine and glutamic acid, were added to filtered-autoclaved lake water as a nitrogen source. The enzymatic activities in both PLM and PRM cells were stimulated by most amino acids tested although the response varied between PLM and PRM cells. Leucine and glutamine amendment gave rise to a larger increase in activities compared with those on other amino acids. On the other hand, viable counts gradually declined after an initial increase in PLM cells (Fig 4.4C, 4.4D).

Fig 4.4 The effect of nitrogen sources on alkaline phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium or phosphate -rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms. Various nitrogen sources were added to the flasks and all flasks were incubated in the dark at 30°C.

A. C. Phosphate-limited medium grown cells

B. D. Phosphate-rich medium grown cells

Log viable count represents cfu ml⁻¹.

Fig 4.4

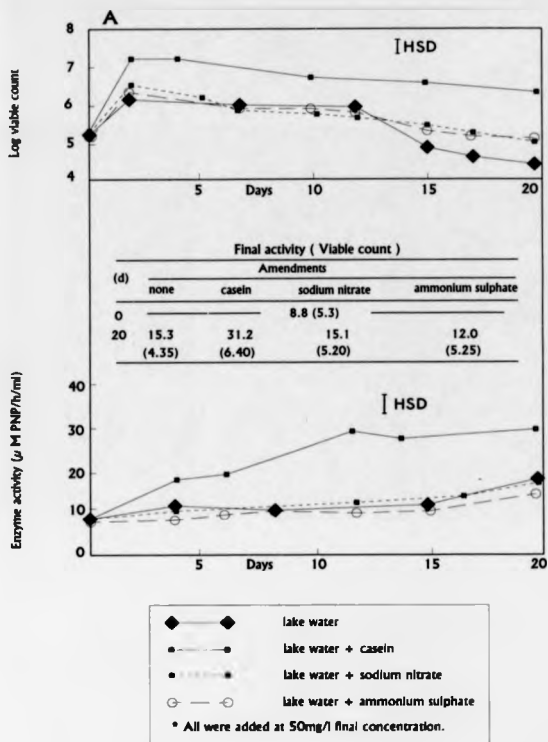


Fig 4.4

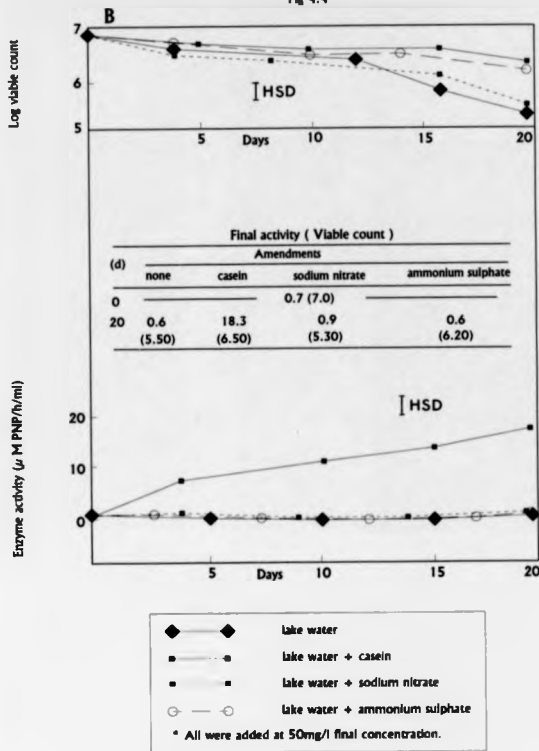


Fig 4.4

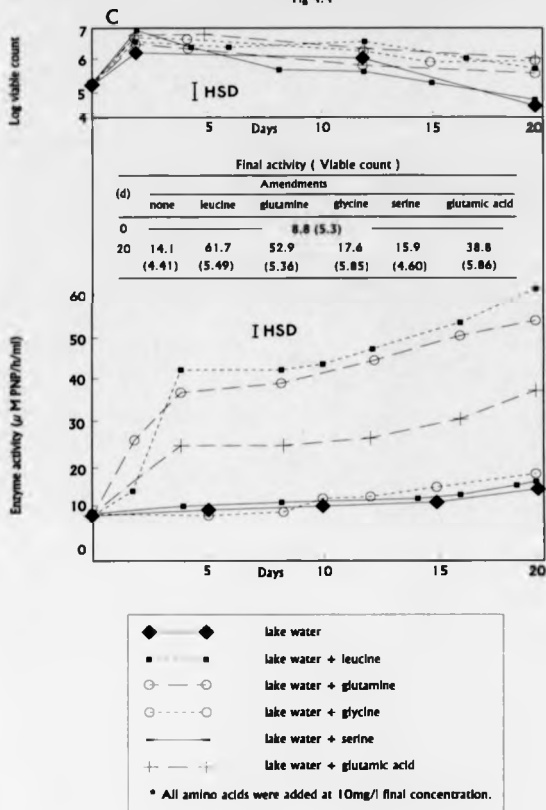
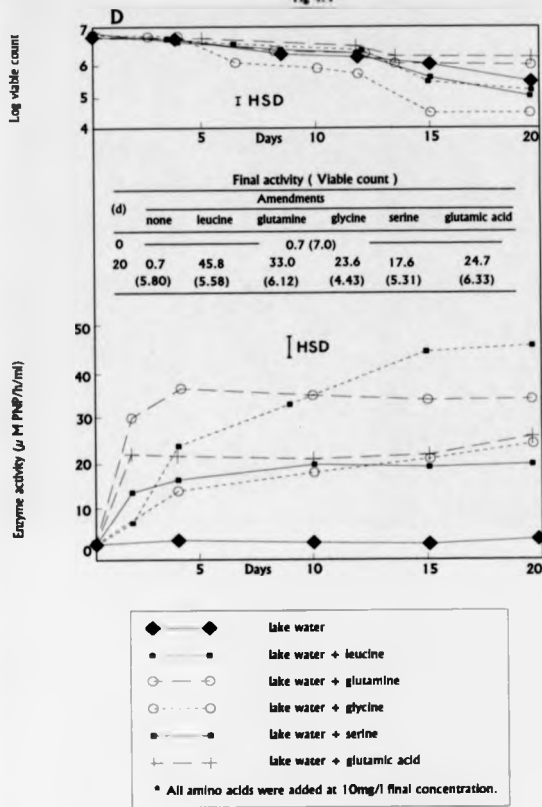


Fig 4.4



4.2.5 The effect of osmotic shock on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Different concentrations of NaCl were used to osmotically shock the cells. The increase in enzyme activity was dependent upon the concentration of NaCl in microcosms containing PLM cells. Although the highest activity was found at the highest concentration of NaCl, viable counts had decreased to zero at 5 to 7% NaCl. No significant difference in viable count was observed at 0 to 1.5% NaCl. There was little production of alkaline phosphatase activity in PRM cells at NaCl concentrations of 0 to 7% (Fig 4.5).

4.2.6 The effect of the osmoprotectant, betaine, on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Betaine was added to filtered-autoclaved lake water containing 3% NaCl to examine its effect on enzymatic activity. Alkaline phosphatase increased in the presence of betaine up to day 12. There was no significant variations during the remainder of the 28 d starvation period in both PLM and PRM cells (Fig 4.6). Betaine as expected led to a longer survival with both PLM and PRM cells.

4.2.7 The effect of oxyanions on alkaline phosphatase activity of *A. hydrophila* in lake water microcosms

Several oxyanions such as phosphate, vanadate, tungstate and molybdate, at a final concentration of 10^{-3} M were added to filtered-autoclaved lake water containing D-glucose at 0.5 g-C/l. Neither the viable count nor the enzymatic activity were significantly influenced by tungstate and molybdate compared to the control. A slight inhibitory effect was exerted by vanadate. However

Fig 4.5 The effect of osmotic shock on alkaline phosphatase activity of *A.hydrophila*

A.hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred filtered-autoclaved lake water microcosms containing different concentrations(w/v) of NaCl. All flasks were incubated in the dark at 30°C.

A. Phosphate-limited medium grown cells

B. Phosphate-rich medium grown cells

Log viable count represents cfu ml⁻¹.

Fig 4.5

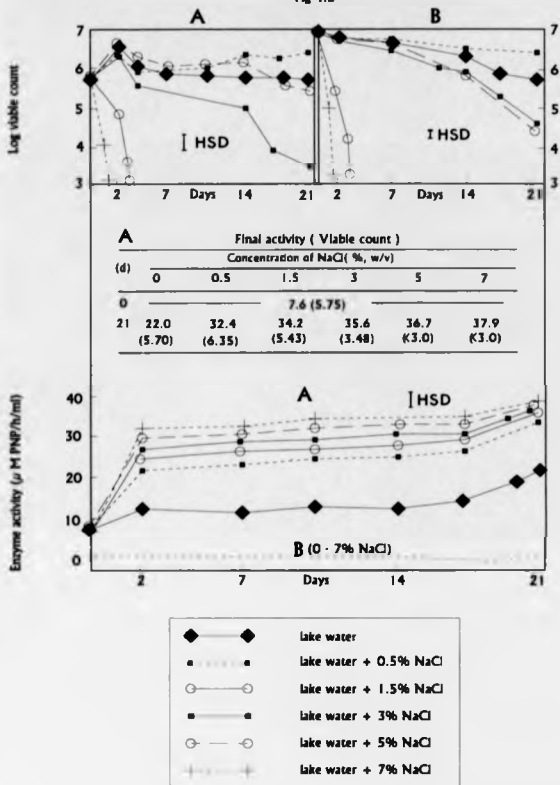


Fig 4.6 The effect of the osmoprotectant, betaine, on alkaline phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtrated-autoclaved lake water microcosms containing NaCl at a final concentration of 3%(w/v). Betaine (5mM) was added to the flasks and all flasks were incubated in the dark at 30°C.

A. Phosphate-limited medium grown cells

Lake water + 3% NaCl + 5mM betaine

B. Phosphate-rich medium grown cells

Lake water + 3% NaCl + 5mM betaine

C. Phosphate-limited medium grown cells

Lake water + 3% NaCl

D. Phosphate-rich medium grown cells

Lake water + 3% NaCl

Log viable count represents cfu ml⁻¹

Fig 4.6

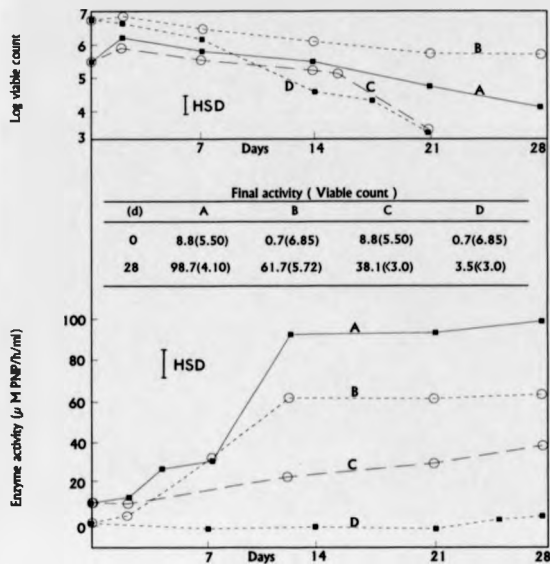
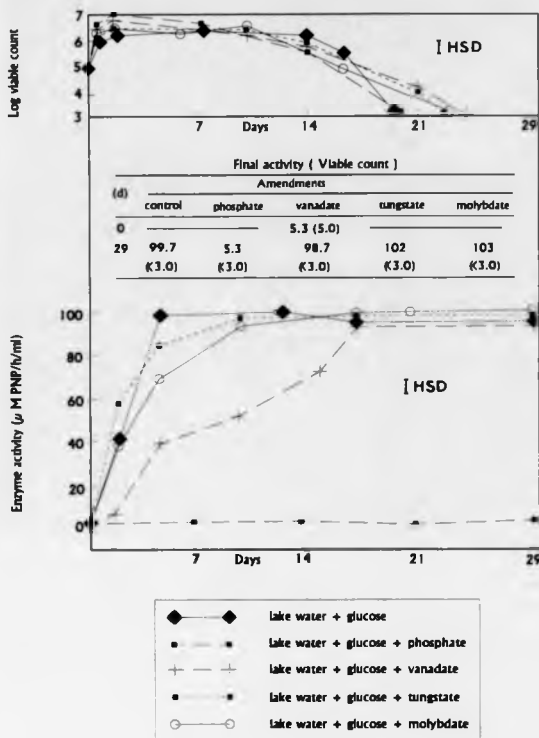


Fig 4.7 The effect of oxyanions on alkaline phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium at 30°C then transferred to filtered autoclaved lake water microcosms containing D-glucose (0.5 g-C/l). Various oxyanions at a final concentration of 10^{-3} M were added to the flasks and all flasks were incubated in the dark at 30°C. Log viable count represents cfu ml⁻¹.

Fig 4.7



phosphate amendment led to a nearly complete inhibition for enzymatic activity (Fig 4.7). Although vanadate did not repress the synthesis of alkaline phosphatase it is known that vanadate at 10^{-3} M causes greater than 90% inhibition of alkaline phosphatase activity in assays of cell-free extracts, and phosphate, the more common competitive inhibitor of alkaline phosphatase causes less than 20% inhibition of enzyme activity at the same concentration (Flint - personal communication).

4.2.8 The effect of carbon sources on acid phosphatase activity of *A. hydrophila* in lake water microcosms

Various carbon sources were used to examine their effects on the acid phosphatase activities of PLM and PRM cells. At 4°C the addition of all the carbon sources to PLM cells except lactose led to an increase in enzyme activity of 2.6 to 3.2 times by day 15 compared to 1.5 times in the control, with a small increase in viable count. A similar increase in activity of 1.5 to 2.6 times compared to the control with PRM cells, was seen with no significant increase in cell population. However the enzyme activity was increased only slightly by lactose amendments in both PLM and PRM cells (Fig 4.8A, 4.8B). At 30°C the acid phosphatase activities in PLM cells increased 3.2 to 6.8 times with all carbon sources, including lactose, compared to a 1.5 times increase in the control by day 15. Viable counts also increased over the first 2 days and then showed a slow decline. A similar effect of carbon sources on acid phosphatase was observed with PRM cells, with a gradual increase in activity of 2.8 to 5.3 times, viable counts remained relatively constant, compared to a 1.5 times increase in the control (Fig 4.8C, 4.8D). At 37°C there was an increase in acid phosphatase activity with all the carbon sources except lactose, which showed very little increase, compared to the control, with both PLM and PRM cells although the cell numbers rapidly declined (Fig 4.8E, 4.8F). The enzymatic

Fig 4.8 The effect of carbon sources on acid phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms. Various carbon sources were added to the flasks and these were incubated in the dark at different temperatures.

Phosphate-limited medium grown cells

- A. 4°C
- C. 30°C
- E. 37°C

Phosphate-rich medium grown cells

- B. 4°C
- D. 30°C
- F. 37°C

All carbon sources were added at 0.5g-C/l final concentration.

Log viable count represents cfu ml⁻¹.

Fig 4.8

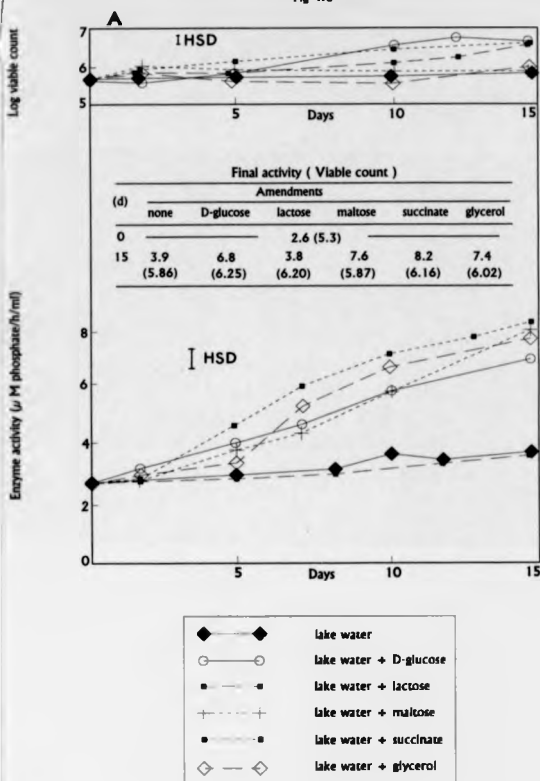


Fig 4.8

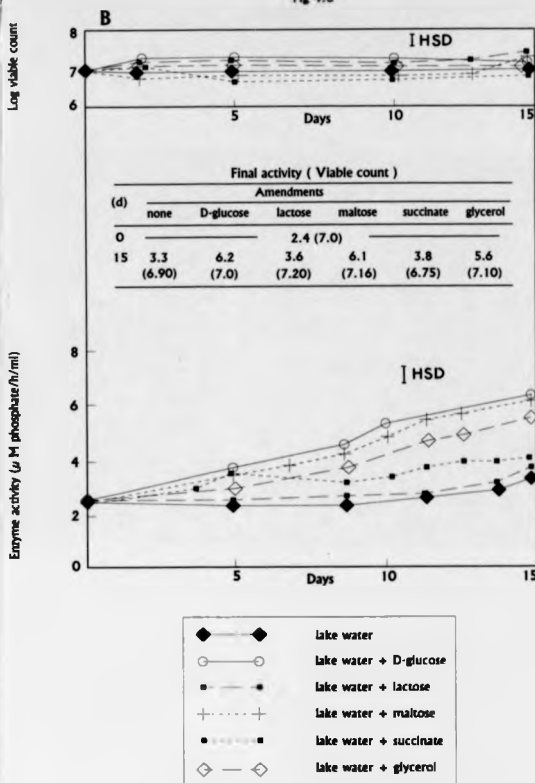


Fig 4.8

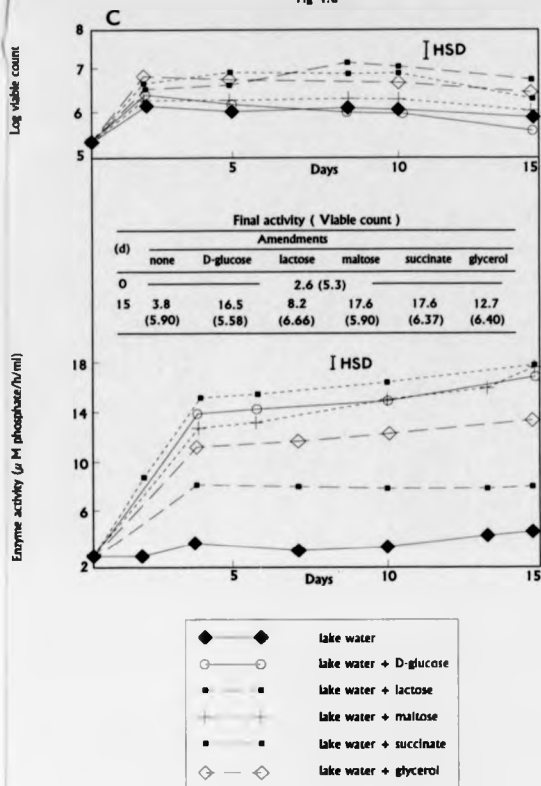


Fig 4.8

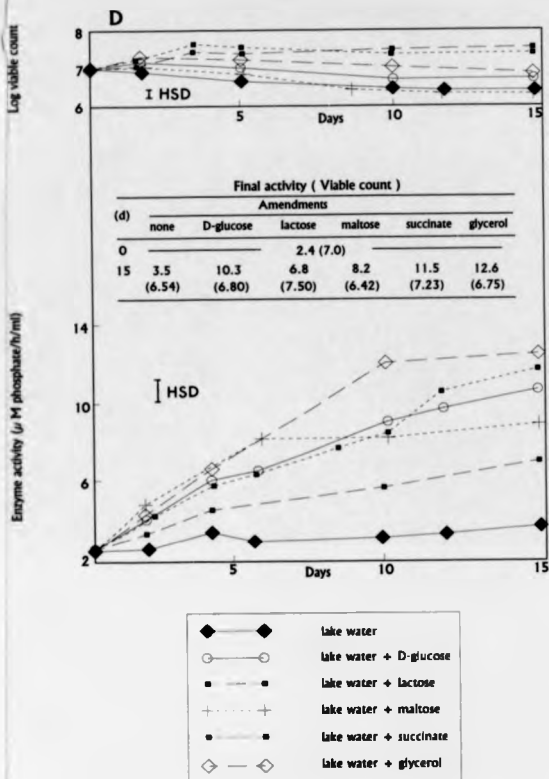


Fig 4.8

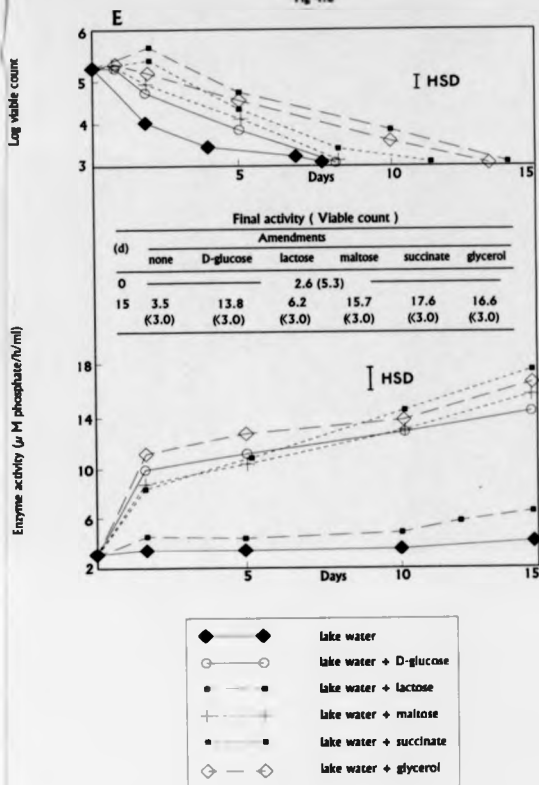


Fig 4.8

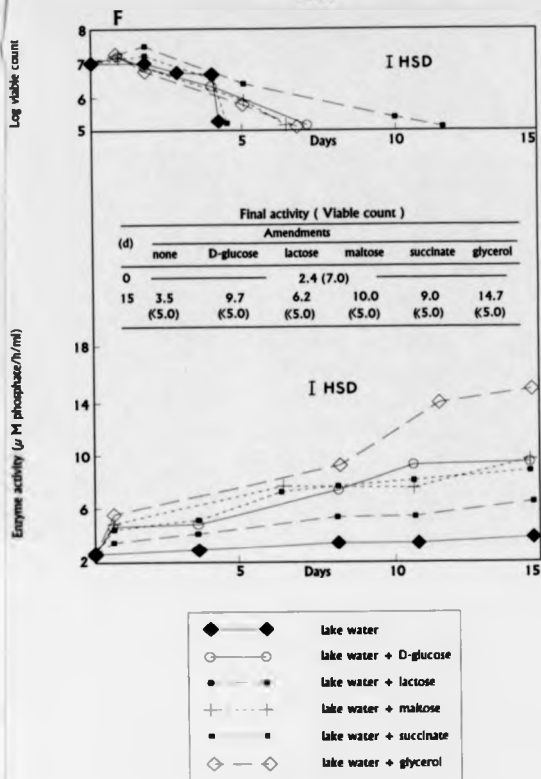


Table 4.2 The effect of carbon sources on acid phosphatase activity of *A. hydrophila* in lake water microcosms at 15 ° and 25 °C

Temp.	Amendment	Enzyme activity (μM phosphate /ml/h)		Log viable count/ml	
		Day 2	Day 15	Day 2	Day 15
15°C (PL)	None	2.6	3.5	5.50	5.78
	D-glucose	6.3	>17.6	6.80	5.94
	Glycerol	4.1	15.9	6.27	5.46
	Maltose	4.2	15.6	6.61	5.64
	Lactose	2.6	5.9	6.09	6.16
	Succinate	5.3	13.6	6.91	4.62
15°C (PR)	None	2.3	4.8	7.04	6.98
	D-glucose	5.1	11.2	7.20	7.11
	Glycerol	5.1	9.8	7.25	6.85
	Maltose	4.4	7.6	7.15	7.06
	Lactose	2.3	5.1	7.27	6.05
	Succinate	3.3	6.5	7.22	5.80
25°C (PL)	None	2.9	3.7	6.0	5.13
	D-glucose	11.5	>17.6	6.21	5.30
	Glycerol	7.3	>17.6	6.15	5.90
	Maltose	15.6	>17.6	6.15	6.0
	Lactose	3.8	11.2	6.53	5.77
	Succinate	14.8	>17.6	6.41	6.61
25°C (PR)	None	2.6	3.5	7.02	6.25
	D-glucose	7.0	14.1	7.37	6.50
	Glycerol	5.0	14.7	7.17	6.66
	Maltose	6.2	14.4	7.47	6.85
	Lactose	3.9	8.8	7.65	5.85
	Succinate	5.7	13.5	7.52	7.15

Key PL : Phosphate-limited minimal medium grown cells
 PR : Phosphate-rich medium grown cells
 Initial population size PL : 5.30 log cfu/ml
 PR : 7.0 log cfu/ml
 Initial enzyme activity PL : 2.6 μM phosphate/ml/h
 PR : 2.4 μM phosphate/ml/h

HSD

Temp.	HSD in Enzyme activity	HSD in Log viable count
15° C, PL	± 2.1	± 0.11
15° C, PR	± 1.6	± 0.14
25° C, PL	± 3.4	± 0.21
25° C, PR	± 2.3	± 0.09

activities at temperatures, such as 15° and 25°C, were also increased in the presence of carbon amendments, except lactose, at 15°C which showed a little increase in activity in both PLM and PRM cells. Viable counts increased over the first 2 days and, then showed no significant fluctuations or a gradual decrease during the starvation period (Table 4.2).

4.2.9 The effect of nitrogen sources on acid phosphatase activity of *A. hydrophila* in lake water microcosms

The addition of casein to filtered-autoclaved lake water microcosms led to an increase, to some extent, in acid phosphatase activity of both PLM and PRM cells. There was also an increase in the viable count of PLM cells but little change in PRM viable count. However sodium nitrate and ammonium sulphate had no apparent effect on acid phosphatase activity (Fig 4.9A, 4.9B). The addition of leucine, glutamine and glutamic acid to PLM cells resulted in an acid phosphatase activity by 5.6, 3.3 and 2.7 times respectively by day 14 compared to a 1.5 times increase in the control. Little or no effect was seen with glycine and serine addition. Viable counts increased over the first 2 days but remained relatively constant over the next 12 d. In PRM cells a similarly increased effect on enzymatic activity was seen with leucine, glutamic acid, glutamine and glycine with up to 4.7, 4.6, 3.9 and 2.5 times increase respectively by day 14 compared with a 1.5 times increase in the control. There was very little change in the viable count over the starvation period (Fig 4.9C, 4.9D).

4.2.10 The effect of phosphate and betaine on acid phosphate activity of *A. hydrophila* in lake water microcosms

The addition of Na_2HPO_4 at a final concentration of 1 mg/l to filtered-autoclaved lake water microcosm containing sodium chloride had little effect on

Fig 4.9 The effect of nitrogen sources on acid phosphatase activity of *A.hydrophila*

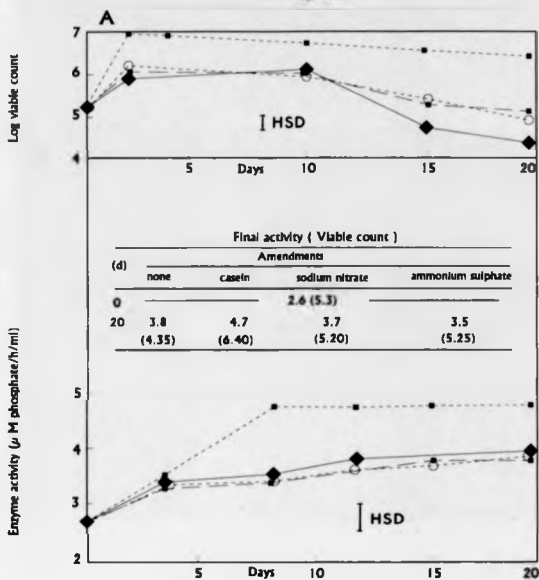
A.hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms. Various nitrogen sources were added to the flasks and these were incubated in the dark at 30°C.

A.C. Phosphate-limited medium grown cells

B.D. Phosphate-rich medium grown cells

Log viable count represents cfu ml⁻¹.

Fig 4.9



- ◆—◆ lake water
- lake water + casein
- - -○ lake water + sodium nitrate
- lake water + ammonium sulphate

* All were added at 50mg/l final concentration.

Fig 4.9

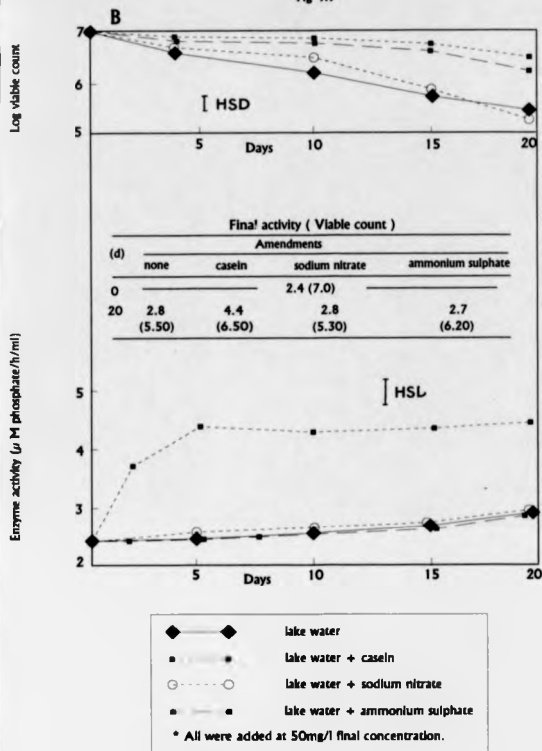


Fig 4.9

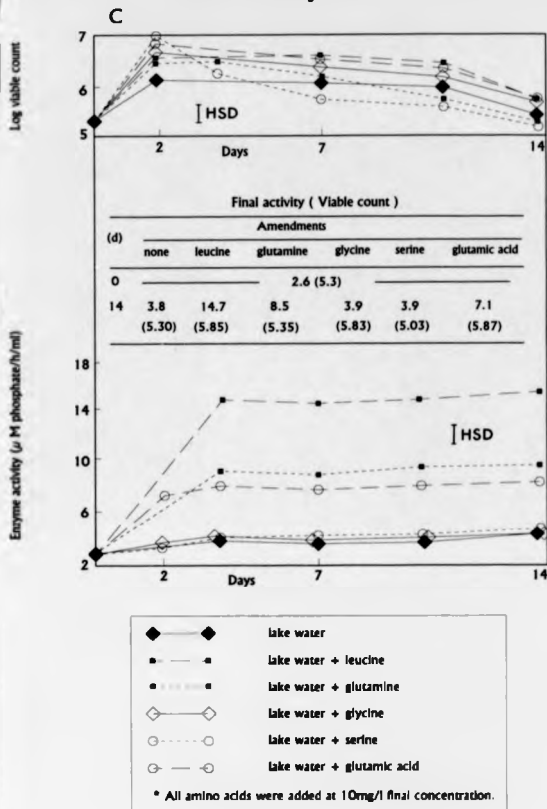


Fig 4.9

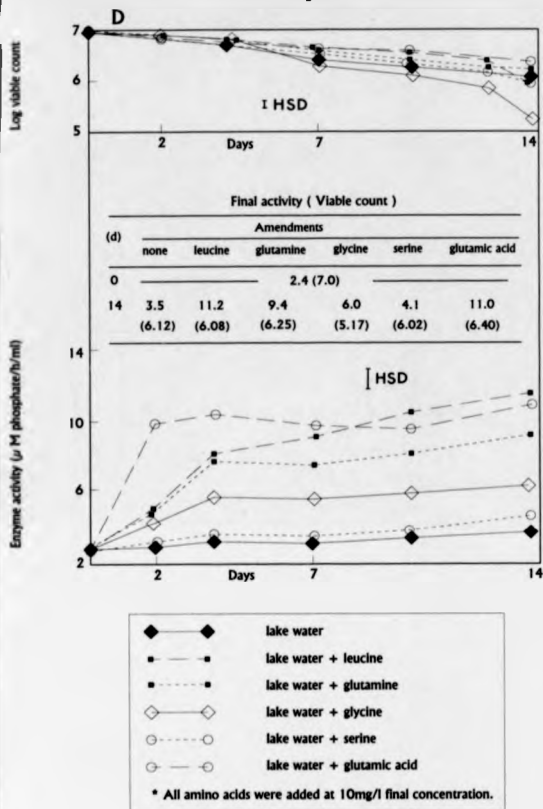


Fig 4.10 The effect of phosphate and betaine on acid phosphatase activity of *A. hydrophila*

A. hydrophila was grown in phosphate-limited minimal medium or phosphate-rich medium at 30°C then transferred to filtered-autoclaved lake water microcosms containing 3%(w/v) NaCl. Phosphate and betaine were added to the flasks and all flasks were incubated in the dark at 30°C.

A. Phosphate-limited medium grown cells

B. Phosphate-rich medium grown cells

Log viable count represents cfu ml⁻¹.

Fig 4.10

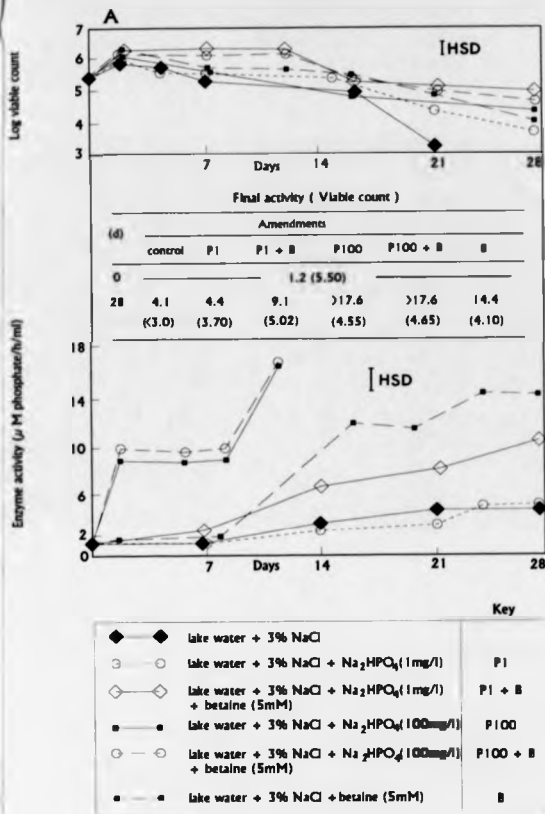
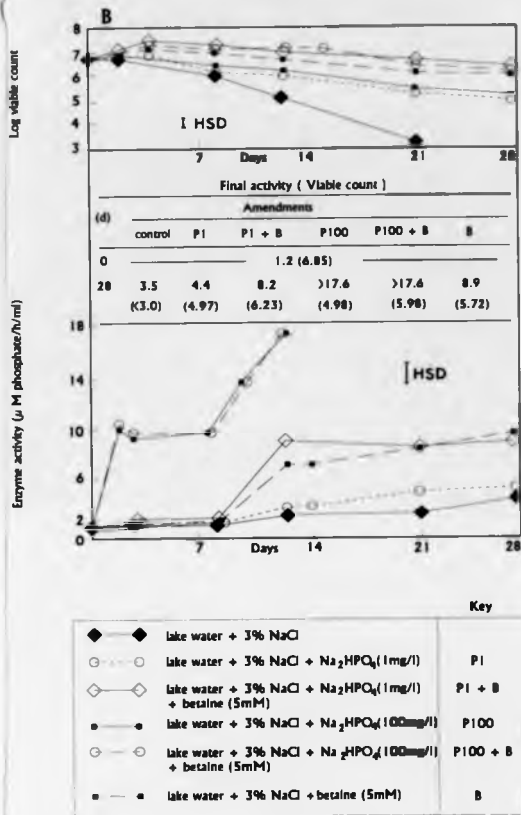


Fig 4.10



acid phosphatase activity in PLM cells whereas the activity increased up to 7.6 times with Na_2HPO_4 (1 mg/l) plus betaine (5 mM), but up to 12 times with betaine (5 mM) only, compared to 3.4 times in the control. The addition of Na_2HPO_4 at 100 mg/l and Na_2HPO_4 (100 mg/l) plus betaine (5 mM) increased acid phosphatase activity significantly up to 17.6 times by day 12. Essentially the same results as shown in PLM cells were observed in PRM cells except that the activities were little different between Na_2HPO_4 (1 mg/l) and Na_2HPO_4 (1 mg/l) plus betaine (5 mM). The viable counts in PLM and cells were slightly increased on betaine amendment but were little changed after phosphate addition (Fig 4.10A, 4.10B).

4.3 Discussion

Throughout this chapter enzyme activity has been recorded as $\mu\text{M pNP}$ released $\text{h}^{-1} \text{ml}^{-1}$ using a constant volume from the microcosm as the sample for the phosphatase assay. An alternative unit of measurement of enzyme activity would have been to use $\mu\text{M pNP}$ released $\text{h}^{-1}(\text{biomass})^{-1}$. Here some measure of the amount of biomass in a sample would be required. Usually this is taken to be viable count, total count or some chemical measure of biomass, such as protein concentration. Matavulj and Flint (1987) for instance used viable count as a means of calculating changes in alkaline phosphatase activity in a pond water sample over a period of time and Flint and Hofton (1976) used optical density measurements to calculate specific activity of alkaline phosphatase for growing cultures of *Pseudomonas* species.

In this chapter using viable count as a measure of activity could have given erroneous results in some cases. For instance in situations in which the viable count was decreasing as the bacteria become stressed but where there was no similar decline in phosphatase activity, this would lead to an increase in specific activity whereas using the measurement used in this chapter would show a constant phosphatase activity. The biomass method could lead to the interpretation of the data as showing depression occurring whereas the volume method indicates no loss of enzyme activity as the viable count declines but crucially for the interpretation of the effects of stress on enzyme activity would show no increase in phosphatase activity through stress.

As total counts were not recorded for all experiments in this chapter this could not be used as a means of biomass. However previous work in Chapter 3, Ozkanca (1993), Lim and Flint (1989) and Flint (1987) has shown that the total count remains constant even when the viable count is declining below the limits of detection. Hence a constant volume of sample from the microcosm would be

expected to contain a constant number of bacteria making the activity measurement used here comparable to a specific activity measurement.

Protein concentration could also have been used as a measure of biomass but as a number of authors have shown the protein concentration of cells also declines as bacteria are starved (Amy & Morita, 1978; Reeve *et al.* 1984a). Using a constant volume is the only reliable method of being able to compare activity in a number of different situations. Obviously three scenarios are possible in these experiments where there has been an increase in the viable count recorded;

1. an increase in viable count and a parallel increase in activity: new biomass produces as much enzyme (i.e. derepression occurs in new biomass in the microcosm.
2. an increase in viable count with no increase in activity: new biomass does not produce new enzyme (i.e. repression has occurred)
3. an increase in viable count and a decrease in activity: the enzyme is being denatured as the bacteria grow.

Similarly the same scenarios could be envisaged for cases where the viable count is constant or decreasing. These situations have been discussed more fully in the text, but obviously an increase in activity as viable count is decreasing is only explained by non-culturable cells still being able to carry out enzyme synthesis. It is these latter cases where the viable count is declining that necessitate the use of a constant volume of sample as a measure of specific activity rather than one of the more common biomass measurements.

The capability of bacteria to synthesize protective proteins or to repair damage through enzyme activity is definitely of importance to a cell which is subjected to unfavorable environmental conditions such as starvation and osmotic stress. Four phosphatases have been identified in the periplasmic space of *E. coli* which can be distinguished from each other by their pH optima for activity and their substrate specificities (Dvorak *et al.*, 1967; Hafckenscheid, 1968; Dassa

et al., 1980). Torriani (1960) suggested that alkaline and acid phosphatases differ in their substrate specificity. The alkaline phosphatase appears to hydrolyze to the same extent monohexophosphates; mononucleotides and synthetic phosphomonoesters. The specificity of the acid phosphatase seems to be confined mostly to the hexose phosphates. The alkaline phosphatase is the enzyme, which plays an important role in phosphate metabolism, and is located in the periplasmic space of the cell. The native alkaline phosphatase is well characterized both structurally and enzymologically (Kim & Wyckoff, 1991). The enzyme alkaline phosphatase of *E. coli* can generate inorganic phosphate from a variety of phosphorylated derivatives. The synthesis of this enzyme is regulated by end-product repression, i.e., the addition of phosphate to the medium inhibits expression of the structural gene, *phoA* (Brickman & Beckwith, 1975). Alkaline phosphatase in measurable amounts is only produced when inorganic phosphate is limiting in the growth medium, such as lake water, whereas acid phosphatases are involved in increasing the availability of hexoses and other carbon sources to the organism either from external sources or from existing internal hexose phosphate pools.

In this chapter, the activity of alkaline and acid phosphatase was determined to investigate the effects of environmental factors, such as starvation stress, osmolarity and so on. This study has shown that the alkaline phosphatase was produced measurable amounts, only by cells grown in a phosphate-limited medium (PLM) and then starved in lake water at different temperatures and not by cells grown in a phosphate-rich medium (PRM). Here, derepression of alkaline phosphatase activity by PLM cells took place with no increase in cell numbers in filtered-autoclaved lake water for 36 d. This increase in enzyme activity is considered to be most probably an adaptation process to starvation conditions. Further the derepression of this enzyme does not seem to be linked to growth but rather to survival of the *A. hydrophila* cells. Some nutrient sources play an important role in the production of alkaline and acid phosphatase

in both PLM and PRM cells. There was, in addition, evidence which indicated that temperature can modify the influence of nutrients on alkaline and acid phosphatase production. Other workers have shown similar results that alkaline phosphatase synthesis in *E. coli* is normally induced by phosphate deprivation in growth medium (Echols *et al.*, 1961; Hofstein, 1961). This, however, is the first report of a derepressible alkaline phosphatase in *Aeromonas hydrophila*. Gauthier *et al.* (1987) observed that some enzyme activities, particularly alkaline phosphatase, significantly increased in cells maintained in nutrient-free sea water, but there are no literature reports of alkaline phosphatase being synthesized in response to starvation stress in lake water.

The results obtained here demonstrate that alkaline phosphatase activities in PLM cells are dependent upon incubation temperatures. The maximal level of enzymatic activity was obtained at 30°C with little fluctuations in cell numbers (after initial growth) over a 36 d starvation period. The activity was lower at temperatures below the optimum growth temperature but there were no significant differences in the viable counts over the count at 30°C, suggesting that the optimal growth temperature in PLM cells provides the best condition for the production of the alkaline phosphatase of *A. hydrophila*. In addition the enzyme activity was increased at 37°C despite a sharp decline in viable counts, indicating that alkaline phosphatase is still stable and produced even at temperatures above the growth optimum during the 36 d starvation period. However PRM cells displayed nearly no derepression of alkaline phosphatase activity in lake water regardless of the temperatures. Alkaline phosphatase production is therefore repressed when the cells are grown in a medium with excess inorganic phosphate and this phosphate must be retained intracellularly.

Gugi *et al.* (1991) investigated the effects of growth temperature on several phosphatase activities of *P. fluorescens* and demonstrated that the activity of three periplasmic phosphatases was maximal at a growth temperature of 17.5°C ; a temperature below the optimal growth temperature. Chemostat experiments

showed that it was the growth temperature itself and not the value of the growth rate that regulated phosphatase activity. They concluded that growth temperature exerts a specific control upon the activity of certain enzymes, such as phosphatase, and that the control within the range of normal growth is probably different from a cold shock or heat shock response. On the other hand Villarejo *et al.* (1983) suggested that the growth rate could be a factor which influences the expression of *phoA*, thus affecting alkaline phosphatase synthesis.

A study, which examined the effect of alkaline phosphatase activity on *E.coli* survival by Gauthier *et al.* (1990), demonstrated that *E.coli* 4100 cells with high alkaline phosphatase activity survived in sea water for longer periods than cells with low or no activity. However mutant cells totally lacking alkaline phosphatase activity held in a phosphate-containing medium before transfer to sea water showed survival almost as high as the wild type strain, indicating that alkaline phosphatase was not the only factor influencing survival. Alkaline phosphatase activity also increased the protection afforded by glycine betaine to osmotically shocked cells. Dassa *et al.* (1982) postulated that the acid phosphatase synthesis of *E.coli* was turned off in the exponential phase of growth and shown to take place exclusively in the stationary phase of the growth. This suggests that it is involved in cell survival rather than cell growth. This enzyme behaves like a group of proteins which included degradative enzymes, such as proteases (John *et al.*, 1978) and the cyclic phosphodiesterase (Anraku, 1966), and also amphibolic enzymes (Goldie & Sanwal, 1980) or truly anabolic enzymes, such as those involved in glycogen biosynthesis (Bridger & Paranchych, 1978).

As shown in chapter 3, some nutrient amendments such as carbon sources, and nitrogen sources including amino acids, increased survival times of *A.hydrophila* in filtered-autoclaved lake water microcosms. The same nutrient sources therefore were used to examine their effects on alkaline and acid phosphatase activities during *A.hydrophila* survival in starved lake water

microcosms. The results suggested that derepression of both alkaline and acid phosphatase was primarily a function of organic carbon content added to filtered-autoclaved lake water containing PLM and PRM cells. The influence of carbon sources, such as maltose, D-glucose, succinate and glycerol, on alkaline and acid phosphatase production in lake water was considerable for both PLM and PRM cells. Moreover the enzyme activities at low temperature, 4°C and at supramaximal temperature, 37°C, were considerably higher in carbon amended microcosms despite a rapid decrease in viable counts, especially at 37°C. These results are similar for those reported for the changes in alkaline phosphatase activity in *E. coli* (Ozkanca, 1993). He also showed that derepression of alkaline phosphatase activity could occur in filtered-autoclaved lake water with *E. coli* rather than with *A. hydrophila*. In both cases derepression of alkaline phosphatase occurs in a starvation medium where little if any growth occurs. Usually the synthesis of alkaline phosphatase is associated with the growth of the microorganism in a growth medium with a limiting phosphate concentration.

Generally the derepressible effect on alkaline and acid phosphatase activity caused by the addition of carbon sources was dependent upon the incubation temperatures. An observed increase in cell numbers was also greater at higher temperature for both PLM and PRM cells. However, the addition of lactose to lake water microcosms led to a relatively delayed derepression in the alkaline and acid phosphatase activity compared with other carbon amendments at all temperatures tested with both PLM and PRM cells in spite of a large increase in viable count. The enzyme activities were highly dependent upon both the incubation temperature and the nutrient source, such as the carbon source, which is a reflection of the fact that temperature and nutrients are primary factors in the synthesis of enzymes. Hence these factors seem to regulate the production of alkaline and acid phosphatase in PLM and PRM cells in starved lake water.

The experimental results, in addition, showed that amino acids, such as leucine, glutamine and glutamic acid, also increased the production of alkaline and acid phosphatase even though viable counts declined slowly. These amino acids could act as a nitrogen source and therefore could be an important factor in the derepression of alkaline and acid phosphatase in starved cells. The occurrence of high levels of derepression of acid phosphatase in *E. coli* was reported after the addition of nutrient supplements, such as casamino acid, succinate and Bacto-peptone (Dvorak *et al.*, 1967), and succinate and glycerol (Von Hofsten, 1961). In this work, the derepression of enzyme activity after the addition of some nutrient sources to starved lake water microcosms is probably concerned with the ability of nutrient sources to maintain the metabolic capability of the cells which were involved in survival. Again with *E. coli* the addition of nitrogen sources caused an increased survival time for *E. coli* (Lim & Flint, 1989) but without increasing alkaline phosphatase activity. In this case the increased survival was not associated with synthesis of phosphate scavenging enzymes. The increase in phosphatase activity with *A. hydrophila* was much more marked than with *E. coli* (Ozkanca, 1993).

When *A. hydrophila* was exposed to osmotic stress in filtered-autoclaved lake water microcosms, the increase in alkaline phosphatase activity in *A. hydrophila* grown in phosphate-limited medium was proportional to the increase in osmolarity ranging from 0 to 7% sodium chloride. However there was no increase in activity in cells grown in phosphate-rich medium. This suggested that osmolarity did not cause derepression of alkaline phosphatase but led to further synthesis of the enzyme if this was already derepressed. Alternatively the increase could be an artefact caused by the osmolarity affecting the outer membrane permeability of the *A. hydrophila* thus allowing easier access for the substrate to the periplasmic space where the alkaline phosphatase is located. Changes in permeability would be shown by an increase in activity. Similar results have been obtained with *E. coli* (Ozkanca, 1993). Again

increasing salt concentration led to an increase in alkaline phosphatase activity even in cases where the viable count fell beyond the limits of detection. In this experiments there was no evidence that the cells were lysing as the total count as determined microscopically remained constant. The increase in activity was much more marked with *A. hydrophila* than with *E. coli* with activity increasing from 4 to 60 μM pNP released $\text{ml}^{-1} \text{h}^{-1}$ with *Aeromonas* and from 0.7 to 1.9 μM released $\text{ml}^{-1} \text{h}^{-1}$ with *E. coli*. Villarejo *et al.* (1983) showed that the synthesis of alkaline phosphatase in *E. coli* varied with changes in the osmolarity of the growth medium, with *phoA* expression increasing with increasing osmolarity. The addition of betaine led to a further increase in alkaline phosphatase activity even in phosphate-rich medium grown cells as well as phosphate-limited medium grown cells. This increase in enzyme activity indicates that betaine, which is an analogue of the amino acid glycine, appears to stimulate the derepression of the enzyme activity further in a similar manner to some amino acids. This is the opposite situation to that recorded for *E. coli* (Ozanka, 1993). In his experiments the addition of betaine to osmotically stressed *E. coli* caused a decrease in alkaline phosphatase derepression. This different response could be due to the ability of *Aeromonas* to metabolise betaine (although this possibility was not reflected in any increase in biomass or viable count during these starvation experiments) or in the ability of betaine to function more efficiently as an osmoprotectant in *A. hydrophila* than in *E. coli*. The possibility that betaine could be incorporated into the cell, given that *A. hydrophila* is a very versatile organism (Van der Kooij *et al.*, 1980) is worthy of further investigation. If betaine were to be used then this could be an explanation for the large increase in activity on alkaline phosphatase seen in samples to which betaine has been added in particular in the case of the high phosphate grown cells where the metabolism of betaine would be expected to create phosphate depleted conditions inside the cell which would be seen by the derepression of alkaline phosphatase as seen here.

Several oxyanions were also added to the starvation medium. Phosphate showed a strong inhibitory effect on alkaline phosphatase activity and vanadate exerted, to some extent, an inhibitory effect although both led to an increase in the viable count compared to the unamended control. Certain oxyanions, such as phosphate and vanadate, can therefore act as very powerful inhibitors on the formation of alkaline phosphatase of *A. hydrophila* in lake water. The results for the effect of phosphate and betaine on acid phosphatase activity showed greatly stimulated enzymatic activity with phosphate at a concentration of 100 mg/l and with betaine, or with both together, indicating that inorganic phosphate and betaine are probably efficient inducers for acid phosphatase production. Acid phosphatase was shown to hydrolyze preferentially molecules possessing numerous phosphoanhydride bonds such as inorganic polyphosphate (Dassa & Boquet, 1981) and is usually considered to be insensitive to phosphate repression. These results suggest that environmental factors could alter the acid phosphatase activity of *A. hydrophila*.

Overall alkaline phosphatase activity of *A. hydrophila* was derepressed by stress including starvation and osmotic stress. The addition of nutrients to filtered autoclaved lake water also led to an increase in alkaline phosphatase activity but this could be a reflection of the cells now being in a phosphate limited growth medium when they are attempting to grow on the carbon and/or nitrogen sources supplied to them as amendments. Although there was rarely an increase in the viable count in the microcosm the enzyme activity could still be increased as the cells responded to starvation stress. In the case of cells grown in high phosphate medium with a repressed enzyme this was derepressed under some conditions most notably under osmotic stress in the presence of an osmoprotectant such as betaine. All this suggests as has also been suggested for *E. coli*, that the bacteria can at least synthesise new enzymes although they are unable to increase in numbers in the microcosms.

Chapter 5

The Effect of Environmental Factors on Exoprotease Activity of *Aeromonas hydrophila* in Lake Water Microcosms

5.1 Introduction

Many Gram-negative bacteria secrete enzymes from the cytoplasm into the extracellular medium. These proteins must cross two hydrophobic barriers, the inner membrane and the outer membrane, which are separated by the peptidoglycan-containing periplasm (Pugsley & Schwartz, 1985).

Protein secretion mechanisms in Gram-negative bacteria can be roughly divided into two main pathways (Pugsley, 1988). The first pathway involves the secretion of the exoproteins across inner and outer membranes in a single step, thereby by passing the periplasm. In this case, there is no requirement for a classical signal peptide which mediates the export of envelope proteins across the inner membrane. The second pathway is signal peptide-dependent with a transient stopover in the periplasm. The translocation of the periplasmic intermediate across the outer membrane is thought to be more or less specific for a given protein or group of proteins. The cholerae toxin from *V.cholerae* (Howard & Buckley, 1983) has been proposed to enter the periplasmic space transiently, and specific helper proteins are thought to be required for its translocation across the outer membrane.

Proteases are present in all living organisms. They display a variety of physical functions. Proteases are enzymes that catalyse the hydrolysis of peptide bonds in proteins or peptides. They are either exoproteases, whose actions are restricted to the amino- or carboxy- termini of proteins, or endopeptidases, which cleave inter-peptide bonds. Microbial proteases are predominantly extracellular and can be classified into four groups based on the essential catalytic residue at their active site. They include serine proteases (EC 3.4.2.1), cysteine proteases (also called thiol proteases) (EC 3.4.2.2), and the metalloproteases (EC 3.4.2.4) (Hase & Finkelstein, 1993). *A.hydrophila* produces a variety of extracellular products, including proteases which cause direct tissue damage, enhanced invasiveness, or provision of nutrients from fish and other hosts (Ljungh & Wadstrom, 1982). Ellis *et al.*(1981) showed that protease of *A.salmonicida* was a

major virulence factor and one of the components responsible for furuncle formation and fish death, which are characteristics of fish furunculosis.

Sakai (1985) indicated that the extracellular protease played an important role in supplying *A.salmonicida* cells with available amino acids and that growth was closely associated with protease production. This role is consistent with the conventional understanding of extracellular protease in heterotrophic bacteria. Albertson *et al.* (1990a) reported that the exoprotease activity measured in the filtered supernatant was due to deliberate release rather than the leakage of cell material as starved cells of both *Vibrio* S14 and *Pseudomonas* S9 treated with chloramphenicol did not release exoprotease activity as there was no degradation of hide powder azure caused by the periplasmic proteins, and as a Mu-directed lac operon fusion mutant of *Pseudomonas* S9 which did not excrete extracellular proteases could be isolated. This mutant synthesized cell-bound exoproteases similar to the wild type, but no activity could be detected in the filtered supernatant even after long-term starvation. Therefore, proteins synthesized during starvation were concluded to mediate production and/or active release of exoproteases.

Leung & Stevenson (1988a) suggested that the major extracellular proteases of *A.hydrophila* NRC505 and ATCC7966 are a thermostable metalloprotease and a serine protease respectively. The thermolabile serine protease of *A.hydrophila* is similar to the thermostable metallo-protease of *A.salmonicida*. Either or both the thermostable metalloprotease and thermolabile serine protease can be produced by some strains of *A.sobria* and *A.caviae*. The *A.hydrophila* metalloprotease was shown to have esterase but no cytotoxic activity (Rodriguez *et al.*,1992) and also appeared to establish infection in fish both by overcoming initial host defences and providing nutrients for cell proliferation (Leung & Stevenson, 1988b). Tn-5 induced protease-deficient mutants of an *A.hydrophila* strain that produced a single metalloprotease showed reduced virulence for fish (Leung & Stevenson, 1988b). Many extracellular bacterial proteases are synthesized as inactive precursors with an additional polypeptide segment (propeptide) that is removed from the mature

secreted protein (Wandersman, 1989). Several roles have been proposed for the propeptides of bacterial proteases. The propeptide may function to keep the protease inactive inside the cell, thus protecting the host cell against an "untethered" protease. It may also play a role in the folding of the proenzyme into the proper conformation necessary for activity or the secretion process or it might temporarily anchor the protease to the membrane (Hase & Finkelstein, 1993). Several studies on the regulation of exoprotease synthesis in Gram-negative bacteria demonstrated the presence of different mechanisms: catabolite repression (Albertson *et al.*, 1990; Boethling, 1975), repression by casamino acids (Albertson *et al.*, 1990) and induction by amino acids (Pugsley & Schwarz, 1985).

It is well established that growing cells of *E. coli* K-12 degrade their bulk protein at an average rate of about 1 to 2% per hour and that, when the cells are subjected to starvation for carbon, nitrogen, or inorganic nutrients, this rate increases to 4 to 5% per hour (Goldberg & John, 1976). Reeve *et al.* (1984a) reported that when *E. coli* culture was starved carbon, glucose, 50% of the cells lost viability in about 6 days. When a K-12 mutant lacking five distinct peptidase activities, CM89, was starved in the same manner, viability was lost much more rapidly; 50% of the cells lost viability in about 2 days, whereas a parent strain lacking only one peptidase activity lost 50% viability in about 4 days. Compared with the wild-type strain and with its parent strain CM17, CM89 was defective in both protein degradation and protein synthesis during carbon starvation. Similar results were obtained with starved *S. typhimurium* LT2 and LT2-derived mutants lacking various peptidase activities. These results indicate that peptidase activities are important to the survival of *E. coli* and *S. typhimurium* cells during carbon starvation. Yen *et al.* (1980) also showed that peptidases play an important role in recovery of *S. typhimurium* from nutritional shift-down. Thus, the peptidases are important to the survival of cells during nutrient limitations or deprivation, or both conditions that normally result in enhanced rates of protein degradation. This chapter reports the survival characteristics and enzyme activity of *A. hydrophila*

under a series of stress conditions. The effect of starvation and the amendment of starvation medium on the activity of the exoprotease of *A. hydrophila* has also been followed.

5.2 Results

5.2.1 The pH profile of an exoprotease of *A. hydrophila*

The pH profile of an extracellular protease of *A. hydrophila* in lake water microcosm is shown in Fig 5.1. There was a broad range of activity over a pH range between 4 and 11.

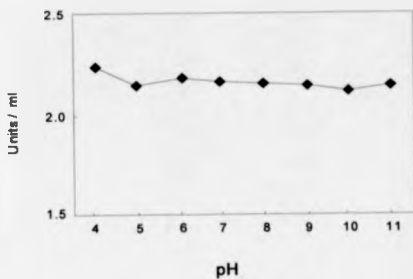
5.2.2 The effect of temperature on exoprotease activity of *A. hydrophila* in lake water microcosms

The exoprotease activity increased with increasing temperature between 4°-30°C. The highest activity was detected at 30°C and the lowest at 4°C. At lowest temperature there was little increase in the activity over the starvation period. Although activity increased over the starvation period, cell numbers of *A. hydrophila* decreased over the same period, with the largest decrease at the highest temperature. At 37°C, above the maximal growth temperature of this organism there was a small increase in activity initially but this fell back to the initial level as the viable count declined (Fig 5.2)

5.2.3 The effect of carbon sources on exoprotease activity of *A. hydrophila* in lake water microcosms

The effect of various carbon sources on exoprotease production was investigated. The addition of D-glucose at a concentration of 50 to 500 mg-C/l to lake water had a positive effect on the production of exoprotease. The increase in activity was proportional to the concentration of D-glucose above 10 mg-C/l D-glucose, a concentration which had no effect on activity. There was no significant

Fig 5.1 The effect of pH on exoprotease activity of *A. hydrophila*



Exoprotease activity was determined at the different pHs with 0.2 M TTA buffer using azocasein as the substrate in the culture supernatant of *A. hydrophila* grown in nutrient broth with shaking at 30°C overnight.

Fig 5.2 The effect of temperature on exoprotease activity of *A.hydrophila* in lake water microcosms

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. All flasks were incubated in the dark at different temperatures. Log viable count represents colony forming unit (cfu ml⁻¹).

Fig 5.2

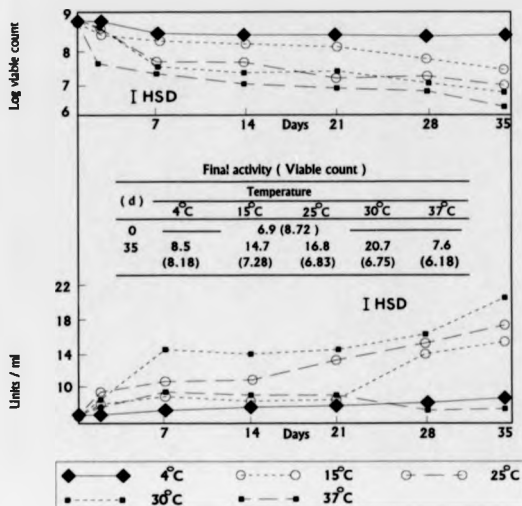


Fig 5.3 The effect of carbon sources on exoprotease activity of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. Various carbon sources were added to the flask and all flasks were incubated in the dark at 30°C. Log viable count represents cfu ml⁻¹.

Fig 5.3

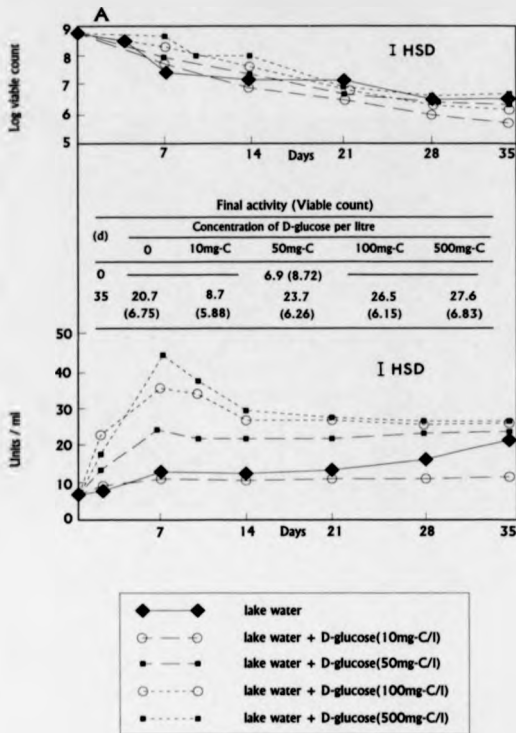
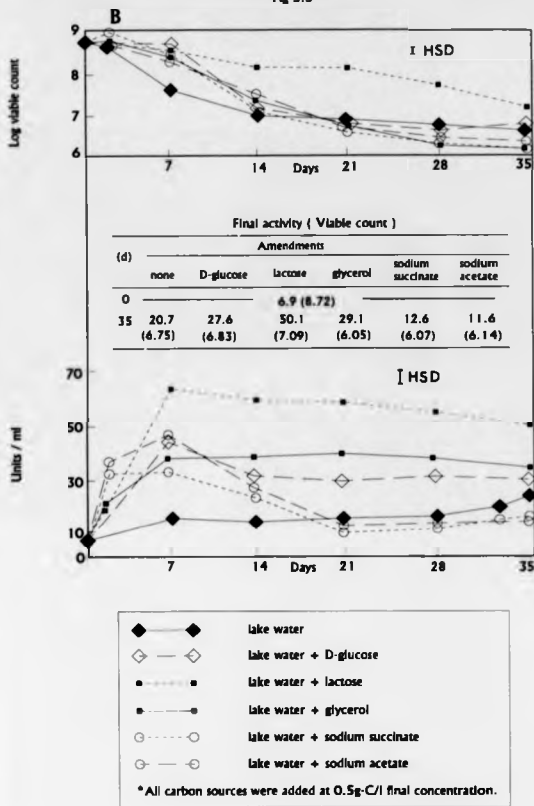


Fig 5.3



increase in cell numbers after the addition of glucose at 10 to 100 mg-C/l compared with that in the control. However the t₉₀ value was increased significantly by the addition of 500 mg-C/l of D-glucose (Fig 5.3A). All the readily available carbon sources tested here also had an effect on the production of exoprotease activity. In particular a marked increase in exoprotease activity was detected after the addition of lactose. This also led to an increase in the viable count and prolonged survival of *A. hydrophila* over the starvation period. The other carbon sources also led to a slight increase in the viable count and increased the t₉₀ value over that for the control although there was little difference in the effect of glycerol, glucose, succinate and acetate (Fig 5.3B).

5.2.4 The effect of nitrogen and phosphorus sources on exoprotease activity of *A. hydrophila* in lake water microcosms

The exoprotease activity of *A. hydrophila* was examined after the addition of various nitrogen sources, such as casein, ammonium sulphate, or sodium nitrate, or the addition of a phosphate source. The addition of ammonium sulphate and phosphate to lake water led to a small increase in protease activity over the starvation period but rather lower activity of protease was observed after sodium nitrate amendment compared to the unamended control. Viable count also decreased in these amended flasks compared to the control over the 28-day period. However exoprotease activity was increased when casein was added to lake water as a nitrogen source. The viable count was also maintained at a higher level than that in the control through the starvation period (Fig 5.4A). The investigation of the effect of individual amino acids on exoprotease activity showed that *A. hydrophila* produced relatively higher amounts of exoprotease when the lake water was supplemented with alanine. Higher enzyme activity was also seen glycine, proline and serine amended samples, whereas lower activity was found with glutamine and glutamic acid amended samples after an initial small increase in

Fig 5.4 The effect of nitrogen and phosphorus sources on exoprotease activity of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. Various nitrogen sources and phosphate were added to the flasks and these were incubated in the dark at 30°C. Log viable count represents cfu ml⁻¹.

Fig 5.4

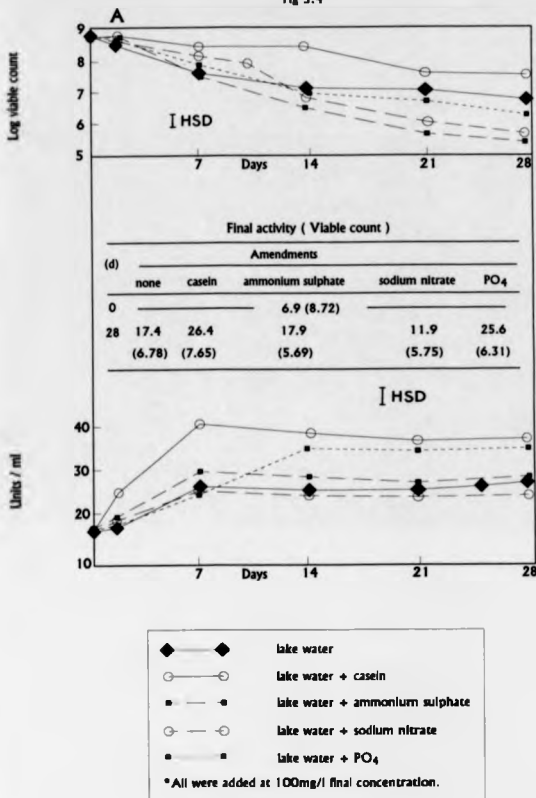
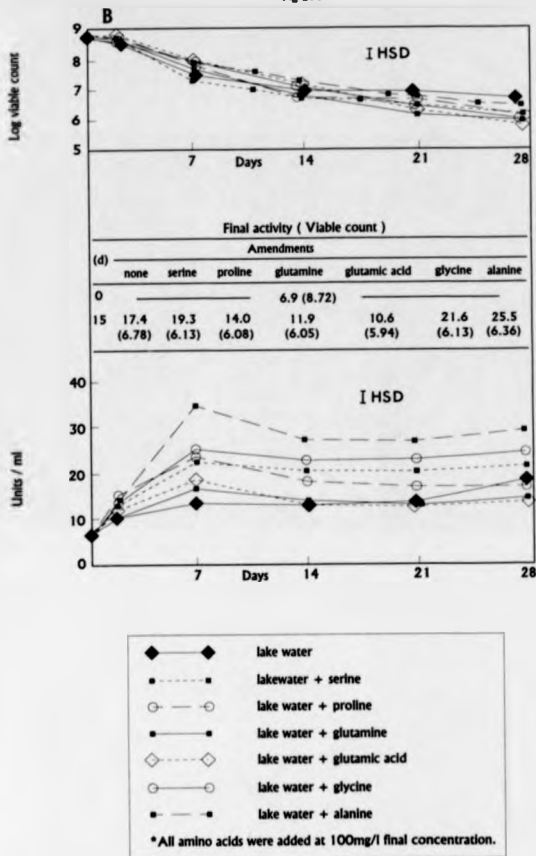


Fig 5.4



activity. There was little difference in the viable counts in the amended and unamended flasks although the control viable count was slightly higher after the 28-day starvation period (Fig 5.4B).

5.2.5 The effect of nutrient deprivation on exoprotease activity of *A.hydrophila* in minimal medium

Both nitrogen and phosphorus starvation led to a gradual increase in exoprotease production compared to the control although there was no significant difference in viable count over the control. Indeed the activity in both cases increased significantly although the viable count declined. Little effect on enzyme production was detected when cells were subjected to carbon starvation and there was a faster decline in viable count compared with the control (Fig 5.5)

5.2.6 The effect of osmoprotectants on exoprotease activity of *A.hydrophila* in lake water microcosms

Osmoprotectants, such as betaine and proline, were added to lake water containing NaCl to examine their effects on exoprotease production by *A.hydrophila*. The addition of 0.5% (w/v) NaCl to lake water led to an increase in protease activity of nearly 2 times that in the control. The viable count also increased as did the survival time. However 3% (w/v) NaCl or 3% NaCl plus proline produced little change in exoprotease activity over the starvation period compared to the control. However there was a large decrease in the viable count with amended flasks than in the control. The highest increase in exoprotease activity was found in lake water microcosms containing 3% NaCl plus betaine, or betaine plus proline, as osmoprotectants. Within 1 week, exoprotease activity increased by up to 8 times of that in the control in both microcosms. This was followed by gradual decline during the remainder of the 35 day starvation period.

Fig 5.5 The effect of nutrient deprivation on exoprotease activity of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to minimal medium, minimal medium minus D-glucose for carbon starvation, minimal medium minus ammonium sulphate for nitrogen starvation, or minimal medium minus KH_2PO_4 for phosphorus starvation. All flasks were incubated in the dark at 30°C. Log viable count represents cfu ml⁻¹.

Fig 5.5

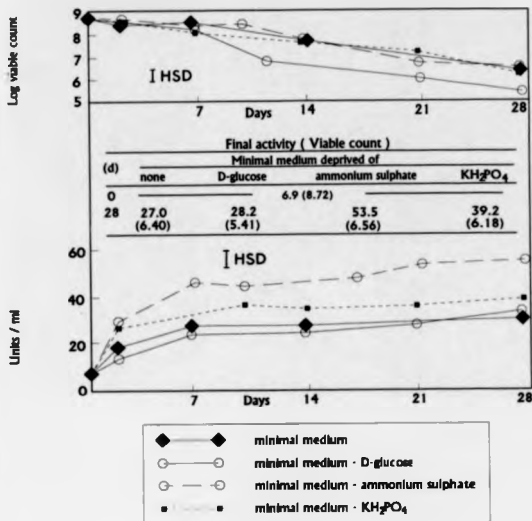
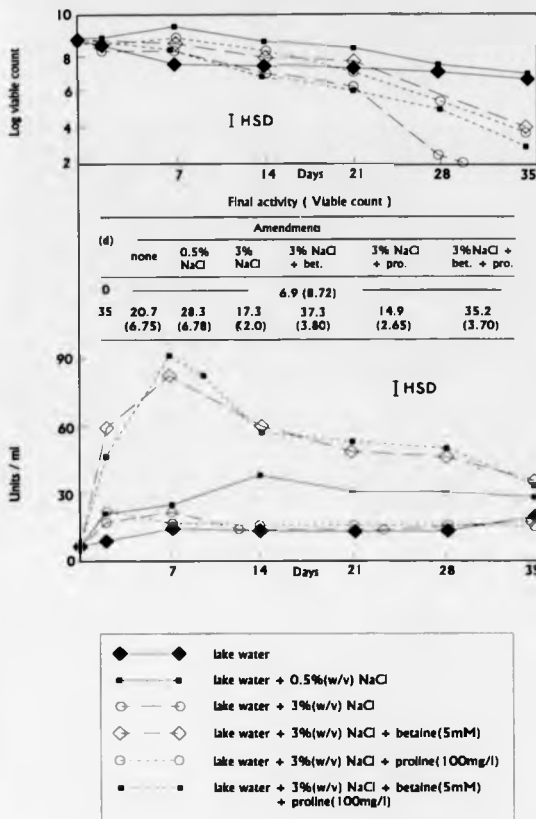


Fig 5.6 The effect of osmoprotectants on protease activity of *Ahydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms with or without NaCl. Osmoprotectants were added to the flasks and these were incubated in the dark at 30°C. Log viable count represents cfu ml⁻¹.

Fig 5.6



This remarkable stimulation in exoprotease activity was probably due to betaine alone as proline has previously been shown to increase activity only slightly (Fig 5.4B). In both cases *A. hydrophila* viable count was slightly higher than the control until day 18 and then declined faster (Fig 5.6), although the decrease was less than that in the flask with only NaCl and no osmoprotectant added.

5.3 Discussion

As in chapter 4, enzyme activity is expressed as activity units ml^{-1} . The same justification of using this measurement of activity applies. The measurement of exoprotease activity in *A. hydrophila* makes the use of protein as a measure of biomass even more unlikely to be a success. This enzyme is synthesised to degrade extracellular proteins to enable the microorganisms to scavenge for traces of nitrogen and carbon sources not available to other bacteria.

In aggregates or microniches where surfaces provide for accumulation of substrate, intense nutrient cycling takes place. This can be attributed partly to the exoprotease activity of the microorganisms associated with these surface (Goldman, 1984). Many proteases are cell bound, but a number of exoproteases have been shown to be produced, especially in the aquatic environments (Somville & Billen, 1983). Although it would not seem beneficial for microorganisms to excrete proteins, especially during nutrient limited conditions, the formation and release of exoproteases could be advantageous for microniche-associated bacteria because the release of extracellular proteases would allow substrate scavenging over a large area (Albertson *et al.*, 1990). O'Reilly & Day (1983) demonstrated that *A. hydrophila* UV108 required the proper pH, temperature, and aeration for optimal protease production. In this chapter, exoprotease activity was determined to examine the effect of long-term starvation stress, nutrient sources, nutrient deprivations and osmotic stress on enzyme activity in *A. hydrophila* in filtered-autoclaved lake water. The results here showed that the exoprotease activity, which has a broad pH range by *A. hydrophila*, was derepressed under starvation conditions in lake water microcosms for 35 d at a range of temperature from 15° to 35 °C even though viable counts of *A. hydrophila* continued to decrease during the starvation period. Further the derepression of an exprotease activity was dependent upon temperature. As the temperature increased to the optimum growth

temperature, there was an increase in the activity of *A. hydrophila* exoprotease in starved lake water.

Similar results were shown by the study of Juffs (1976), who showed that temperature and composition of the medium could influence markedly the production of a protease by *Pseudomonas* spp. In his work, protease production per unit of growth fell as the temperature of incubation was lowered from the optimum for enzyme production and also sharply at temperatures above the optimum temperature for growth, despite extensive growth. Protease production was not, therefore, related directly to the amount of growth over a range of incubation temperatures.

When *Vibrio* sp. strain SA1 was stressed to grow at a low rate, protease production was abundant (Wiersma & Harder, 1978). Similarly when *A. hydrophila* UV108 was stressed by limiting the carbon or nitrogen source, large amounts of protease were produced, despite very sparse growth. These kinds of stresses may induce the cell to produce a series of scavenging enzymes, such as proteases, the purpose of which is to digest potential polymeric nutrient sources to provide readily metabolizable substrates for microbial growth (O'Reilly & Day, 1983). In addition Albertson *et al.* (1990) demonstrated that exoprotease activity was produced in a marine bacterium during 120 h of total energy and nutrient starvation. Since marine bacteria experience intermittent periods of greatly reduced or totally arrested growth due to nutrient limitation, the degradation of organic polymers via exoprotease activity is an example of how non-growing bacteria possessing high affinity amino acid uptake systems may participate in the cycling of nutrients in the marine environment. Their study exhibited an increase in exoprotease activity after 4h (*Pseudomonas* sp.) or 5h (*Vibrio* sp.) starvation compared with cells at the onset of starvation. In this chapter the activity of exoprotease was studied over a much longer period of time. In all cases the viable count was decreasing throughout the starvation period but there was a continued increase in activity even in the control microcosms which had no other nutrient

amendments made. Over this longer incubation period, usually 35 d, exoprotease activity usually increased dramatically over a period of 5 to 7 days then began to decline. Albertson *et al.* (1990) also showed that the increase in activity of exoprotease in their marine bacterium was also transient. Activity could decrease if the enzyme is unstable and production ceases or if the enzyme is actively being degraded. This latter suggestion seems to be more unlikely suggestion. Alkaline and acid phosphatases in Chapter 4 remained relatively constant as these are known to be stable enzymes. Exoprotease of *A. hydrophila* is less well characterised and no details of its long term stability are available.

The increase in the degradation of hide powder azure after short-term starvation could be due either to *de novo* synthesis of proteins involved in the synthesis and/or active release of exoproteases during the initial reorganizational phase of starvation or an accumulation of exoproteases at the cell surface during starvation. The former hypothesis appears to be correct, since inhibition of exoprotease activity was observed when chloramphenicol was added to cells starved for 5 h. This also indicates that the exoprotease activity found in the filtered supernatant was not due to cell lysis or otherwise passive release of cell material.

It was suggested, by the results obtained here on nutrient amendments, that the addition of carbon, phosphorus, and nitrogen sources to lake water stimulated, for the most part, the production of extracellular protease although the viable counts of *A. hydrophila* gradually decreased over the same time period. A large increase in exoprotease activity was seen, especially with lactose, casein and alanine, with relatively long survival time of the cells in filtered-autoclaved lake water microcosms. O'Reilly & Day (1983) suggested that exoprotease production in *A. hydrophila* UV108 was increased on complex nitrogen and carbon energy sources and larger amounts of protease was produced on poorly utilized carbon sources compared with the amounts produced on more readily utilizable carbon sources. Different effects of nitrogen sources on protease activity were shown in several

studies. *A. hydrophila* produced large amounts of a protease when grown on casein as the nitrogen source, whereas on casamino acids poor enzyme yields resulted (O'Reilly & Day, 1983). Using an ammonium-containing chemically defined medium, Riddle *et al.* (1981) showed that glutamic acid increased both growth of and protease production by *A. hydrophila*. The difference in protease production on different nitrogen sources may reflect differences in the amino acids that induce the protease, especially if the enzyme can exist in both constitutive and inducible forms (O'Reilly & Day, 1983).

It is not really possible to compare the information available for the synthesis of exoprotease activity in complex laboratory media and with the impoverished lake water used here. Even after the addition of a nutrient amendment the lake water would still be depleted for at least one major nutrient source. No complex nutrient addition such as synthetic sewage used previously (Lim & Flint, 1989) was tried here. After the addition of a carbon source such as glucose the water would be nitrogen limited and one might expect that the final activity seen would then be higher than in the case of the addition of casein which would only leave the sample phosphate depleted. However this was not the case and except for the addition of phosphate which would leave the microcosms carbon and nitrogen starved the final activities were very similar after the 35 day incubation period. However the transient activity increase was highest when lactose and alanine were used as the amendments. There is no easy explanation of why two such disparate nutrients should lead to similar increases in activity.

McKellar (1982) also suggested that the difference in protease induction in *P. fluorescens* might be due to variations in the quantity of specific inducer(s) present in the medium. Complex organic nitrogen sources vary in the concentration of free amino acids and small peptide present. Thus, each nitrogen source may have the required amino acids present in (i) amounts insufficient to induce activity, (ii) optimal induction concentrations, or (iii) excessive induction amounts, which would repress protease synthesis; such effects were observed by

Wiersma and Harder (1978). The concentrations of trace elements in the complex nitrogen sources may also cause changes in protease production ; for example, Riddle *et al.* (1981) found Zn^{2+} to be stimulatory and Fe^{2+} to be inhibitory to protease synthesis by *A. hydrophila*. In most gram-negative strains, amino acids have been found to induce exoprotease synthesis. A model explaining this regulation was described by Daatselaar and Harder (1974). Proteins in the environment spontaneously produce a small concentration of free amino acids, which act as a signal to bacteria that proteins are present. As a result exoprotease synthesis is increased. The addition of small quantities of amino acids to protein-containing media was reported to enhance protease production (Litchfield & Teria, 1970 ; Fairbairn & Law, 1987).

McKeller (1982) demonstrated that when skim milk supernatant was fractionated on the basis of molecular weight, all the inducing ability for extracellular protease production by *P. fluorescens* was found in the small molecular weight (<5000) fraction and large molecular weight proteins isolated from skim milk supernatant were ineffective inducers. Juffs (1976) pointed out the importance of small molecular weight compounds in the induction of protease by *P. aeruginosa*. The stimulation or enhancement of protease production when low concentrations of amino acids are added to a protein-containing medium may be due not only to their ability to induce, but also to the action of the elevated levels of protease in releasing more inducing compounds (Fairbairn & Law, 1987).

They also suggested that the regulation of protease production by *P. fluorescens* appeared to be based on a balance between induction by low concentrations of low molecular weight degradation products and sensitivity to end-product catabolite repression. In their study, *P. fluorescens* did not produce extracellular protease when grown in the presence of inorganic nitrogen. Protease was produced in the exponential growth phase when cultures were grown on complex substrates such as protein, but during the early stationary phase when cultures were grown on single amino acids or mixtures of amino acids. This may

be the result of catabolite or end product repression of protease production by relatively high concentrations of the free amino acids during exponential growth. Keen & Williams (1966) showed that high concentrations ($\geq 1\%$, w/v) of glutamic acid apparently repressed protease production of *Pseudomonas lachrymans* and the repression was more pronounced when glutamic acid was supplemented with sucrose or inorganic nitrogen, and suggested, in addition, that rapid assimilation of carbon by the bacteria repressed protease production and counteracted the stimulation of synthesis by organic nitrogen.

Albertson *et al.* (1990) investigated, using *Vibrio* sp., the positive role of cyclic AMP (cAMP) which reverses the repression of exoprotease synthesis by glucose. Ullman & Danchine (1983) suggested that the increase in exoprotease synthesis due to the addition of cAMP is in agreement with the notion that starving cells would benefit from the induction by cAMP of enzymes enabling the cells to utilize a broader range of substrates, since many cAMP-controlled proteins mediate transport or catabolism of carbon substrates. These results also displayed the effects of various stress-conditions, such as nutrient deprivation and osmotic shock on the production of exoprotease by *A. hydrophila*. Increased extracellular protease activity was found as a consequence of nitrogen- and phosphorus starvation in minimal medium despite little difference in cell numbers over the control, indicating that nutrient-stressed conditions probably could induce the production of an extracellular protease by *A. hydrophila*. O'Reilly & Day (1983) demonstrated the production of large amount of protease by *A. hydrophila* under limiting nutrient sources, such as nitrogen or carbon source.

Overall stress has been shown to lead to the increase in exoprotease activity of *A. hydrophila* in much the same way as it leads to an increase in alkaline and acid phosphatase activities in this organism. All these enzymes are produced in order to scavenge for traces of nutrients which cells may require to enable them to survive adverse conditions. Without the ability to scavenge then cells would be unsuccessful in outcompeting producer organisms in the natural environment. The

fact that *A. hydrophila* can survive this type of starvation better than *E. coli* (comparing similar situation used by Ozkanca, 1993) is expected as *E. coli* is not a natural inhabitant of the aquatic environment whereas *A. hydrophila* is and *E. coli* does not produce an exoprotease enzyme. Many disparate additions, for instance lactose, alanine and phosphate, to the lake water led to an increase in exoprotease activity. The only common response seems to be that this enzyme is produced as part of the overall stress response of this bacterium. Any form of starvation stress leads to the production of exoprotease activity. Similarly osmotic shock lead to a transient increase in activity but the addition of the osmoprotectant betaine increased the activity in the presence of osmotic shock considerably. Betaine again may be being utilized as a nitrogen source as in Chapter 4 but this remains to be seen. As with phosphatase enzymes the fact that the organisms are starved does not seem to inhibit the synthesis of enzymes associated with the starvation response of bacteria. One would expect these enzymes used for scavenging for traces of nutrients to be produced in response to starvation stress, but the observation that they are also produced in response to osmotic stress is a novel observation.

Chapter 6

**The Effect of Biotic and Abiotic Factors on Plasmid Transfer from
Aeromonas hydrophila to *Escherichia coli* in Lake Water
Microcosms**

6.1 Introduction

Genes may be transferred horizontally from donor to recipient strains by several mechanisms. Microbial gene transfer mechanisms may have evolved as a means for bacteria to adapt to changing environments and may represent a normal function of bacteria in aquatic ecosystems (Paul *et al.*, 1991). A brief definition of gene transfer mechanisms and a short discussion of how they may operate under environmental conditions follows.

6.1.1 Transformation

Transformation is the process whereby a naked piece of double-stranded DNA is taken up by a competent bacterial cell. A competent cell is one which can be transformed easily. The incoming piece of DNA is integrated into, and then replicated with, the recipient cell genome. Natural or physiological transformation occurs in many genera of bacteria, especially Gram-positive bacteria where the competence state is part of the organisms' life cycle. Competency can be induced in other organisms by treatment with calcium chloride which makes the cell wall permeable to double-stranded DNA and subsequently cells can be artificially transformed or transfected (Davis *et al.*, 1980). Natural transformation in the environment may be facilitated by DNA released by decaying microbial cells or excreted by living cells. This may be especially important in densely populated microniches. The DNA can be protected from nuclease digestion by adsorption to particulates, especially to clays. On the other hand, DNA is very rapidly degraded in some environments, with turnover times as rapid as 6.5 h. It may also become biologically inaccessible through adsorption to particles. Low cell densities, common to many aquatic communities, may further reduce the opportunity of *in situ* natural transformation (Reanney *et al.*, 1983).

6.1.2 Transduction

In transduction, the donor DNA is packed into the capsid of a transducing bacteriophage and injected into the recipient cell upon a subsequent infection. Generalized transduction occurs at low frequency and donor genes are transferred randomly when pieces of the disintegrating donor chromosome are taken up by the maturing phage particles. Lysogenic phages that integrate into a discrete site in the host chromosome may transduce genes which lie next to this site if they are excised imprecisely upon the onset of the lytic cycle. Such events result in the transfer of specific genes; thus, the term "specialized transduction" (Davis *et al.*, 1980). Transduction as a mechanism of gene transfer in the environment seems advantageous because the nucleic acids are protected in the proteinaceous capsid of the virus. In addition, the very strict host range of most phages (with the exception of P1) limits their potential to act as vectors for gene spread (Reanney *et al.*, 1983).

6.1.3 Conjugation

Plasmids can mediate their own transfer from donor to recipient cells by a process which requires cell-to-cell contact. It was first described for the Gram-negative species, *Escherichia coli* (Lederberg & Tatum, 1946). Contact and possibly transfer of the DNA itself occurs through a sex pilus, a filamentous appendage on the donor cell surface which interacts with a specific site on the surface of the recipient cell. Conjugative plasmids carry the transfer operon (*tra*) which codes for all the functions needed for conjugation, including sex pilus formation. The integration of a conjugative plasmid into the donor chromosome by legitimate recombination may lead to the transfer of chromosomal genes (e.g., Hfr transfer mediated by the F factor). Conjugative plasmids may also mediate the transfer of non-conjugative plasmids (these are usually smaller and exist in the

host in a higher copy number than conjugative plasmids) when they both reside in the same donor cell. This process, termed mobilization, occurs either by cointegrate formation between the conjugative and the non-conjugative plasmids, or the non-conjugative plasmid may be transferred via the system coded by the conjugative plasmid. The latter does not involve physical contact between the two plasmids, but it depends on the presence of a mobilization (mob) site on the non-conjugative one (Davis *et al.*, 1980). Theoretically, conjugation could be an efficient mode of gene transfer because plasmids are autonomous replicons, and conjugation is the only gene transfer mechanism that does not require homologous DNA sequences for stable maintenance in the recipient cell.

Plasmids are ubiquitous in a large variety of organisms isolated from diverse environments. Further, many traits which confer advantages in stressed and hostile environments are coded by plasmids (Hardy, 1986). Specific environmental habitats support dense and active microbial communities where the requirement for cell-to-cell contact can be met easily. Transfer by conjugal systems which depend on rigid pili is stimulated on solid interfaces. Therefore, the concentration of bacteria at an interface in the environment seems to be advantageous for gene transfer. However, several considerations suggest that conjugation may not play such an important role in the spread of genes in the environment. Intracellular barriers, such as entry exclusion, possible incompatibility between two plasmids residing in the same cell, and restriction-modification systems which enable the recognition and degradation of incoming donor DNA, act to prevent transfer. In addition, layers of extracellular material, deposited on cells in their natural habitats, may prevent transfer and sex pili may be sensitive to shearing by wave action and currents. Microbes exist in some environments (e.g., oceans, deserts) in low densities which reduce the opportunity of cell-to-cell contact. Finally, although conjugal gene transfer does not depend on cell reproduction, it does depend on the metabolic state of the donors and

recipients. The metabolic rate of organisms in their natural habitats is reduced in response to the scarcity of growth substrates (Reanny *et al.*, 1983).

6.2 Results

6.2.1 Cell concentration and plasmid used

The mating mixtures with a donor, *A. hydrophila* (Km^r; carrying a plasmid for kanamycin resistance), and a recipient, *E. coli* nal-R (nalidixic acid resistant), at an initial concentration of 1.28×10^9 and 1.23×10^9 cells/ml respectively, were used for the following experiments investigating the effect of temperature and nutrients on plasmid transfer. The plasmid used in this experiments is a F-like plasmid, whose *tra* regions are similar to that of the F-plasmid, comprising about 21 genes. Many F-like plasmids specify antibiotic-resistance (R plasmids). The most conspicuous features coded for by F-like plasmids are sex pili. These are hollow tubes, composed largely of protein, which protrude from cells with a length of 2-20 μ m and are essential for conjugation (Hardy, 1986). The sex pili (Fig 6.1A) necessary for conjugation between donors and recipients, and the profile of plasmid DNA (Fig 6.1B) isolated from donors and transconjugants are demonstrated. No plasmid transfer was detected in nutrient broth from *E. coli* 2366 (or *E. coli* 2599) carrying an I-like plasmid (specifying kanamycin resistance) to *A. hydrophila* in the first step to produce donor cells, *A. hydrophila* (Km^r).

6.2.2 The effect of temperature on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

The results of the effect of mating temperature on the number of transconjugants and on transfer frequency using lake water microcosm as the mating medium are shown in Fig 6.2. The range of temperatures assayed was from 15 to 37°C. The recovery of transconjugant numbers was observed to be significantly different between the temperature of 30° (or 37°C) and 25°C. The

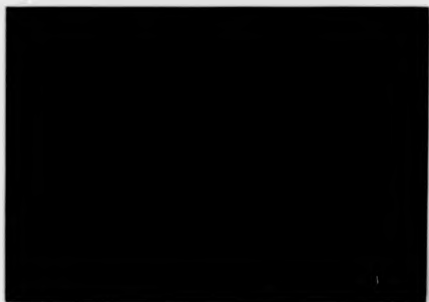
Fig 6.1 Electron micrograph of *A.hydrophila* showing a sex pilus and gel electrophoresis of plasmid DNA

A. Transmission electron micrograph of *A.hydrophila* (donor) shows sex pilus (arrow indicator). The bar represents 1 μ m.

B. Agarose gel electrophoresis shows the profile of plasmid DNA in *A.hydrophila* and *E.coli*. Kanamycin resistance plasmid was initially transferred from *E.coli* 2345 to *A.hydrophila* to produce the donor cells, then plasmid was transferred again from *A.hydrophila* (donor) to *E.coli* nal-R (recipient) to make transconjugants.

- | | |
|--------------|---|
| <u>Lanes</u> | 1. <i>E.coli</i> 2345 |
| | 2. <i>A.hydrophila</i> (donor) |
| | 3. <i>E.coli</i> nal-R (transconjugant) |

A



B



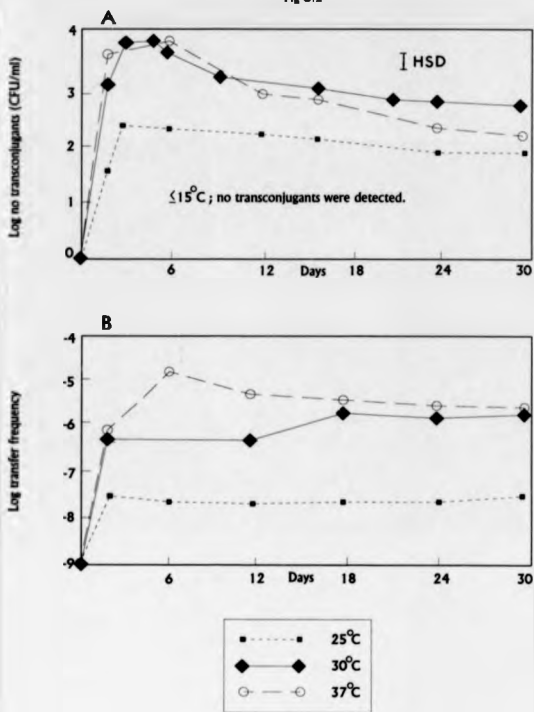
Fig 6.2 The effect of temperature on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

A. hydrophila (Km^r) as a donor and *E. coli* (nal^r) as a recipient were grown in nutrient broth at 30° and 37°C respectively then transferred together to filtered-autoclaved lake water microcosms. These flasks were incubated in the dark at different temperatures.

Transconjugants(A) were detected on nutrient agar plates supplemented with both kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

The frequency of transfer(B) was estimated as the number of transconjugants per number of surviving recipients.

Fig 6.2



number of transconjugants increased to 7.2×10^3 and 7.3×10^3 cells/ml within the first 6 days at 30°C (optimal growth temperature for the donor) and 37°C (optimal growth temperature for the recipient) respectively, which was then followed by decreasing number of transconjugants during the remainder of the 30 day starvation period. A smaller increase up to 2.3×10^2 cells/ml in the number of transconjugants was found within 2 days at 25°C, but the number remained constant during the remainder of the starvation period. No transconjugants were detected at 15°C or less in starved lake water during the 30 day incubation period (6.2A). The frequency of the plasmid transfer increased as the temperature increased such that the frequency of transfer was of the order of $37^\circ > 30^\circ > 25^\circ > 15^\circ\text{C}$. The maximum frequency of transfer was 1.9×10^{-5} per donor at 37°C but 3 logs lower at 25°C. In general transfer frequency reached a constant value after 2 days with no significant variation between day 2 and day 30, the end of the experimental period (Fig 6.2 B).

6.2.3 The effect of carbon and phosphorus sources on plasmid transfer from *A.hydrophila* to *E.coli* in lake water microcosms

The results for the influence of several carbon sources and phosphate at different temperatures on the number of transconjugants obtained are shown in Fig 6.3. The addition of carbon sources such as lactose, glycerol and glucose, and phosphate to lake water microcosms at 30°C had a positive effect, increasing the number of transconjugants to between 1.9×10^4 and 5.2×10^4 cells/ml in the amended flasks compared to 1.6×10^3 cells/ml in the control on day 2. There was then a slow decline in the number of transconjugants, comparable to the decline shown previously for *A.hydrophila* alone. The maximal number of transconjugants were detected with 0.5g-C lactose/l and 100 mg PO_4 /l as amendments. There was also a larger number of transconjugants with glucose amendment of 0.5g-C/l compared to 0.1g-C glucose/l (Fig 6.3A). Carbon

Fig 6.3 The effect of carbon and phosphorus sources on plasmid transfer from *A.hydrophila* to *E.coli* in lake water microcosms

A. hydrophila (Km^r) (donor) and *E.coli* (nal^r) (recipient) were grown in nutrient broth at 30° and 37°C respectively then transferred together to filtered-autoclaved lake water microcosms. Various carbon sources and phosphate were added to the flasks and these were incubated in the dark at different temperatures.

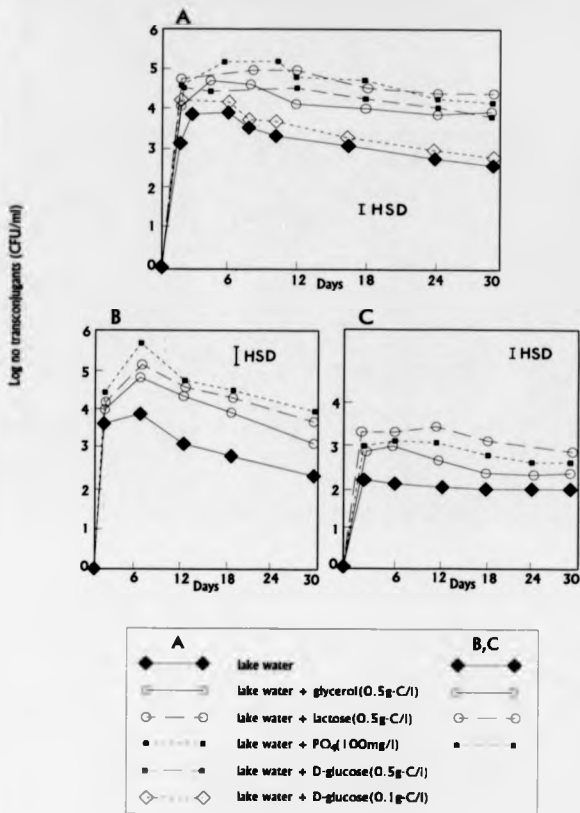
Transconjugants were detected on nutrient agar plates supplemented with both kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

A. 30°C

B. 37°C

C. 25°C

Fig 6.3



amendments, such as lactose and glycerol, and phosphate amendment led to an increase in the number of transconjugants in lake water at 37°C reaching maximal values of between 8.0×10^4 and 1.8×10^5 cells/ml compared to 7.3×10^3 cells/ml in the control by day 6, followed again by a decline to the end of the experimental period (Fig 6.3B). Similar results were exhibited at 25°C (Fig 6.3C).

6.2.4 The effect of nitrogen sources on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

The results corresponding to the influence of nitrogen sources in lake water microcosms at different temperatures on the number of transconjugants obtained are shown in Fig 6.4. Nitrogen sources, such as ammonium sulphate, casein, serine and glutamine at a concentration of 100 mg/l, had a significant effect on the increase of transconjugant numbers in lake water at 30°, 37° and 25°C. The effect was more pronounced at 37° than at 30°C or 25°C. The number of transconjugants detected after nitrogen amendment was between 4.9×10^3 and 1.2×10^5 compared to 1.6×10^3 cells/ml in the control at 30°C on day 2 (Fig 6.4A), between 6.7×10^4 and 2.7×10^5 compared to 7.3×10^3 cells/ml in the control at 37°C on day 6 (Fig 6.4B), and between 6.5×10^2 and 1.5×10^3 compared to 2.5×10^2 cells/ml in the control at 25°C on day 2 (Fig 6.4C).

6.2.5 The effect of synthetic sewage and nutrient broth on plasmid transfer from *A. hydrophila* to *E. coli*

The results corresponding to the influence of different concentrations of synthetic sewage in lake water, and the influence of nutrient broth at different temperatures on the number of transconjugants obtained are shown in Fig 6.5.

Fig 6.4 The effect of nitrogen sources on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

A. hydrophila (Km^r) (donor) and *E. coli* (nal^r) (recipient) were grown in nutrient broth at 30° and 37°C respectively then transferred together to filtered-autoclaved lake water microcosms at different temperatures. Various nitrogen sources were added to the flasks and these were incubated in the dark.

Transconjugants were detected on nutrient agar plates supplemented with kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

A. 30°C

B. 37°C

C. 25°C

Fig 6.4

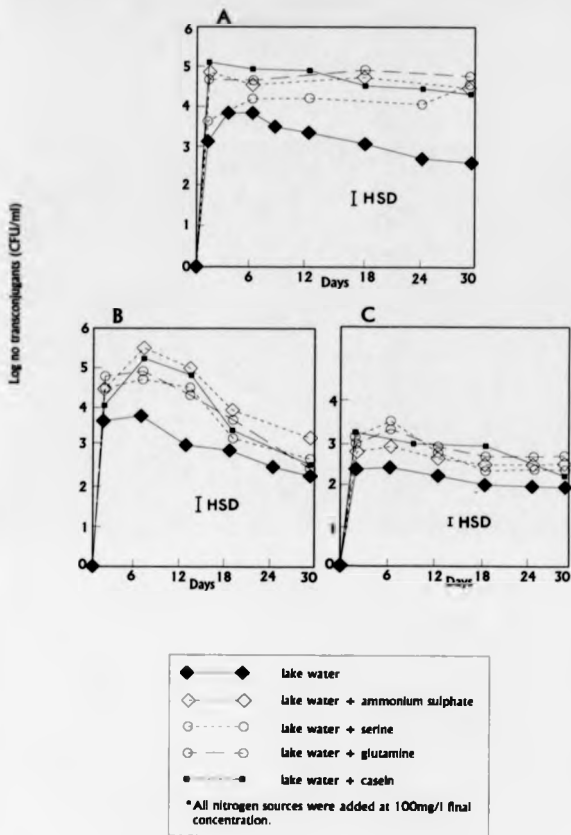
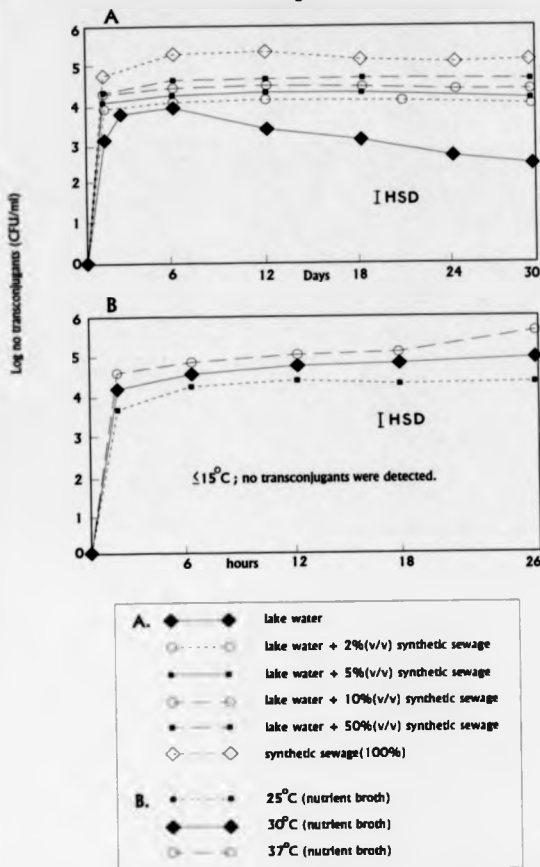


Fig 6.5 The effect of synthetic sewage and nutrient broth on plasmid transfer from *A.hydrophila* to *E.coli*

A.hydrophila (Km^r) (donor) and *E.coli* (nal^r) (recipient) were grown in nutrient broth at 30° and 37°C respectively then transferred together to filtered-autoclaved lake water microcosms with synthetic sewage at 30°C or to nutrient broth medium at different temperatures. All flasks were incubated in the dark.

Transconjugants were detected on nutrient agar plates supplemented with both kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

Fig 6.5



The number of transconjugants recovered was dependent upon the concentration of synthetic sewage added to the lake water at 30°C. The transconjugant numbers were increased as the concentration of synthetic sewage increased from 0% to 100% (v/v), reaching the maximal values of 2.0×10^5 cells/ml at 100% of synthetic sewage compared to 7.2×10^3 cells/ml in the control during the same time period. There was little subsequent decline after an initial increase in numbers was observed with any of the concentrations of synthetic sewage tested (Fig 6.5A). In nutrient broth medium, the number of transconjugants very rapidly increased to day 2 and then progressively increased temperature-dependently in the order of $37^\circ > 30^\circ > 25^\circ\text{C}$ reaching the final value of 2.2×10^5 , 8.0×10^4 and 2.5×10^4 cells/ml respectively by day 26. There was no plasmid transfer observed at 15°C and below in lake water microcosms amended or unamended, or in nutrient broth (Fig 6.5B).

6.2.6 The effect of initial cell ratio (donor to recipient) on plasmid transfer from *A.hydrophila* to *E.coli* in lake water microcosms

The variation in the number of transconjugants depending upon the variation in the ratio of donor (D) to recipient (R) cells was shown with lake water as the mating medium. The number of transconjugants varied significantly with variation of cell ratios and was positively dependent on the concentration of donor cells. The number of transconjugants detected increased as the concentration of donors increased, reaching a maximal value, 3.2×10^4 cells/ml, when the ratio of D : R was 54 : 1 and a maximum, 7×10^1 cells/ml, when the ratio of D : R was 1 : 15 (Fig 6.6).

Fig 6.6 The effect of initial cell ratio (donor to recipient) on plasmid transfer from *A.hydrophila* to *E.coli* in lake water microcosm

A.hydrophila (Km^r) (donor) and *E.coli* (nal^r) (recipient) were grown in nutrient broth at 30° and 37°C respectively then transferred together to filtered-autoclaved lake water microcosms at different donor-to-recipient cell ratios. All flasks were incubated in the dark at 30°C.

Transconjugants were detected on nutrient agar plates supplemented with both kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

Fig 6.6

	Cell ratio (donor : recipient)	Concentration of the donor/ml	Concentration of the recipient/ml
○ — ○	54 : 1	1.28×10^9	2.37×10^7
■ — ■	20 : 1	1.28×10^9	6.40×10^7
○ — ○	5 : 1	1.28×10^9	2.42×10^8
◇ — ◇	2 : 1	1.28×10^9	6.60×10^8
— — —	1 : 1	1.28×10^9	1.23×10^9
■ — ■	1 : 5	2.43×10^8	1.23×10^9
◆ — ◆	1 : 15	8.05×10^7	1.23×10^9

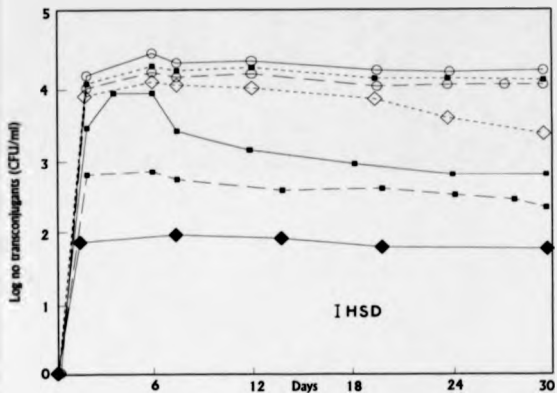


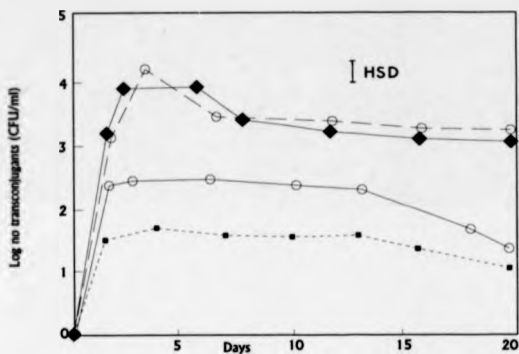
Fig 6.7 The effect of initial cell density on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

A. hydrophila (Km^r) (donor) and *E. coli* (nal^r) (recipient) were grown in nutrient broth at 30° and 37°C respectively then transfer together to filtered-autoclaved lake water microcosms at different cell densities. All flasks were incubated in the dark at 30°C.

Transconjugants were detected on nutrient agar plates supplemented with both kanamycin (50 µg/ml) and nalidixic acid (50 µg/ml).

Fig 4.7

	Approximate concentration of the donor or the recipient /ml	Concentration of the donor/ml	Concentration of the recipient/ml	D : R (approx.)
◆ — ◆	1×10^9	1.28×10^9	1.23×10^9	1 : 1
○ — ○	7×10^8	7.00×10^8	6.60×10^8	1 : 1
○ — ○	3×10^8	2.70×10^8	2.65×10^8	1 : 1
■ — ■	8×10^7	8.30×10^7	8.00×10^7	1 : 1



6.2.7 The effect of initial density on plasmid transfer from *A. hydrophila* to *E. coli* in lake water microcosms

The number of transconjugants produced in lake water microcosm was dependent on the initial cell density of the donor and recipient. The lowest number of transconjugants, 5.0×10^1 cfu/ml, was detected with the lowest cell density, 8×10^7 cfu/ml for both donor and recipient. The number of transconjugants reached a maximal value, 1.3×10^4 cfu/ml, when the cell density was above 7×10^8 cfu/ml for both donor and recipient (Fig 6.7). There was a dramatic increase in the number of transconjugants when the initial cell density was increased from 3×10^8 to 7×10^8 cfu/ml.

6.3 Discussion

Plasmids have been detected in bacteria isolated from a variety of aquatic environments. Frequencies of plasmid incidence in marine isolates range from 23% to 46%. A survey of plasmids in freshwater epilithic bacteria showed that 10-15% of isolates contained at least one plasmid, depending on whether the samples were from unpolluted or polluted sites (Rochelle *et al.*, 1989). Plasmid transfer, although most often associated with conjugation, can also be accomplished by transformation and transduction. Some of small, naturally occurring nonconjugative plasmids of environmental origin have been shown to be transferred by transformation (Rochelle *et al.*, 1988). It is well-known fact that plasmids play an important role in the adaptation of bacteria to their environment. Plasmid-encoded characters include antibiotic and heavy metal resistance, fermentation of sugars and aromatic compounds, toxin production, bacteriocin production, virulence determinants, UV-resistance, and restriction-modification systems (Gordon, 1992).

A plasmid's fitness can be thought of in terms of its ability to be present in future generations of bacteria. The reproductive strategy and success of a plasmid can be divided into a vertical and a horizontal component (Simonsen, 1991). Vertically, the plasmid is maintained in a bacterial population by replicating within its host and partitioning plasmid copies between the daughter cells. Horizontally, it is maintained by transmitting a copy to a new host cell through the process of conjugation or mobilization. The efficiency of the vertical mode of plasmid spread is dependent on the cost of plasmid carriage; synthesizing plasmid DNA and expressing genes of selfish plasmids, which simply parasitizes bacteria, can be costly and may result in a significant reduction in the growth rate of the host (Levin, 1980). The cost of maintaining a plasmid could also have a major impact on the survival of cells under starvation conditions. Gowland and Slater (1984) showed that under starvation conditions

(nutrient limitation) in a chemostat or in pond water that transfer of plasmids and stability of plasmids in *E.coli* species was greatly reduced, and that cells possessing plasmids were lost from a population earlier than those without. Flint (1987) however showed little difference in the survival characteristics of plasmid-plus or plasmid-minus *E.coli* in river or lake water microcosms. The horizontal component is dependent on the density of potential recipients and the rate at which transfer occurs. The plasmid can potentially make up for the vertical losses if the rate of horizontal transfer is sufficiently high (Simonsen, 1991).

The results obtained show that the increase in the recovery of the number of transconjugants and the frequencies of transfer occurred in filtered-autoclaved lake water microcosms and this recovery was in proportion to the temperature. The elevation of temperature up to the optimum gives rise to an increasing transconjugant numbers and frequencies. The results suggest furthermore that the plasmid transfer can take place in starved aquatic environments if the proper temperature is available to the bacteria. There was no detectable number at 15°C or below, suggesting that *in situ* where the lake water temperature will rarely exceed 15°C, plasmid transfer by conjugation between these species would be a rare event. Similarly several investigators found, as a rule, that low temperatures had a negative effect on plasmid transfer, detecting maximum frequencies in the range of 20° to 30°C (Rochelle *et al.*, 1989; Sandt & Herson, 1991). Fernandez-Astorga *et al.* (1992) also have indicated a negative effect of low temperature on plasmid transfer of *E.coli*, as the frequencies detected at low temperatures ($\leq 15^{\circ}\text{C}$) are significantly lower than those detected at the higher temperatures assayed (20°-37°C) although transconjugant formation can occur at 8°C for some *E.coli* species. This is in agreement with Rochelle *et al.* (1989), who reported that conjugation would be more likely to take place at temperatures higher than would normally be expected in temperate aquatic environments.

Singleton & Anson (1981) showed that the conjugal transfer of the F-type R-plasmid R1 *drd-19* in *E.coli* was likely to be determined at least partly by temperature, and that, with no transconjugants being obtained at 15°C during a 48h mating period, the minimum temperature for the transfer of an R plasmid *in vitro* was 17°C. In the experiments in this chapter using the same plasmid, no transfer was observed between *E. coli* and *A. hydrophila* at 15°C and it is possible that the minimum temperature at which this plasmid will transfer is around 17°C as Singleton and Anson (1981) suggest regardless of the bacterial species involved in the mating pair formation. This is consistent with the suggestions that the bacterium has to be metabolically active to be able to transfer a plasmid that *E. coli* and possibly *A. hydrophila* cease to be active enough at this low temperature. This suggests that the conjugal transfer of R plasmids in many British rivers and in British coastal waters would not be precluded on the basis of temperature when temperature is considered as an independent variable because the annual maximum temperature of a typical British river is between 16° and 22°C (Walling & Webb, 1981) and of coastal waters between 17° and 22.2°C (Jones & Swainson, 1980). Bale *et al.* (1988) also suggested that temperature was the dominant factor affecting plasmid transfer between *Pseudomonas* spp. in river water, with a linear relationship between frequency and temperature.

Walmsley (1976) showed no mating-pair formation by *E.coli* below 24°C. Between 30° and 41°C pair formation increased very rapidly, followed by an equally sharp decline between 41° and 45°C. The lack of pair formation at low temperatures could be attributed either to a loss of donor pili or an inability of the donor pili to attach to the recipient cell. Since extreme temperatures modify the physiological state of bacteria, it is also possible that the ability for genetic transfer is affected. Novotny and Lavin (1971) suggested that the number of F pili per cell in *E.coli* varied with the growth temperature, but the average length of F pili remained constant. The maximum number of pili per cell occurred between 37° and 42°C ; below 37°C the number decreased, reaching zero at

about 25°C. When cells were shifted down from 37° to 20°C, outgrowth (assembly) of pili ceased and approximately 50% of the attached pili were released in 2 min.

The results reported here examining the effect of nutrients on plasmid transfer showed that a larger number of transconjugants were detected after nutrients, such as carbon, nitrogen, and phosphorus sources, and nutrient mixtures, such as synthetic sewage and nutrient broth, had been added to the lake water microcosm, compared with those obtained under starvation conditions in unamended controls. In addition the number of transconjugants was positively dependent upon the concentration of synthetic sewage added to the lake water. These observations suggested that plasmid transfer would be significantly influenced by the presence or absence of nutrients in aquatic environments, and nutrient availability can be considered to be one of predominant factors in governing the occurrence of plasmid transfer.

Many authors have shown a close link between the transfer of plasmids and availability of nutrients, where frequency increases along with the nutrient concentration in the medium. Thus Trevors and Oddie (1986) found that plasmid transfer in stream water occurred only when it was amended with dilute nutrient broth. However in this thesis plasmid transfer did occur even in unamended water. Bale *et al.* (1987) demonstrated, using *P. aeruginosa*, that plasmid transfer would be greatest where available organic substrate concentrations are highest, such as directly below sites of organic enrichment. This result was confirmed here, using *A. hydrophila* rather than *P. aeruginosa*, with the highest frequency of transfer occurring with the highest concentration of synthetic sewage. These microcosms would correspond to the sites of organic enrichment shown by Bale *et al.* (1987) to increase plasmid transfer. The increased frequency of transfer and the higher number of transconjugants seen with the addition of inorganic phosphate to lake water shows that it is not simply a case of increasing the organic content of the water which is possibly responsible

for the increased plasmid transfer in the microcosms amended with synthetic sewage. Where inorganic phosphate was used as the amendment no organic supplement was added suggesting that the organic concentration in the water was sufficient to satisfy the organic requirement for transfer. Although the addition of inorganic phosphate to microcosms does not increase the survival times of *A. hydrophila* (Chapter 3) or *E. coli* (Lim & Flint, 1989) in filtered-autoclaved lake water microcosms there must be an increase in cellular metabolic capability after the addition of inorganic phosphate in order to increase the efficiency of transfer of the plasmid between the donor *A. hydrophila* and the recipient *E. coli*. The increase in transfer efficiency after the addition of a single carbon supplement is in line with the need for an increase in the organic component of the water as suggested by Bale *et al.* (1987) and Trevors and Oddie (1986). Similarly the increase after the addition of amino acid or protein amendment, such as casein, could be taken as an increase in organic content but the increase in plasmid transfer efficiency after the addition of ammonium sulphate is harder to explain. However it is the addition of ammonium sulphate which has led to the increased survival of both *A. hydrophila* (Chapter 3) and *E. coli* (Lim & Flint, 1989). The increase in plasmid transfer in all the amended microcosms could be simply a reflection that the addition of any potential nutrients from inorganic phosphate to nitrogen and carbon sources increases the metabolic capabilities of the cells, possibly transiently, and the plasmid is transferred more efficiently as a result. Relatively large increases in the levels of nutrients in the environment have been found to increase the frequency of plasmid mobilization, which varied from 5.2×10^{-6} in drinking water to 5.9×10^{-3} in Trypticase soy broth medium (Sandt & Herson, 1991).

Rochelle *et al.* (1989) suggested that the significant increase of transfer frequencies with added organic matter indicates the physiology of the bacteria involved in conjugation to be an important determinant. Fernandez-Astorga *et al.* (1992) have reported that the concentration of total organic carbon in the

mating medium affects both the number of transconjugants and the transfer frequency for *E.coli*. They also found that plasmid transfer by conjugation can take place even in adverse conditions like the absence of nutrients points out that the availability of nutrients may be considered to be one of the factors of greatest influence on frequency. Jones *et al.*(1991) have further shown that the deprivation of nutrients, in their model aquatic system utilizing lake water, is probably a major reason for reduced plasmid transfer from *P.aeruginosa* to Gram-negative aquatic isolates.

Several workers have shown a direct relationship between the initial density of parent cell and the number of transconjugants detected (O'Morchoe *et al.*,1988 ; Fernandez-Astorga *et al.*, 1992). Fry and Day (1990) have reported that it is very important to avoid using very low initial densities of cells (less than 2×10^5 cfu/ml) or too few donors, as plasmid frequencies drop rapidly below optimum numbers. These factors reflect that the detection of transconjugants in lake water is influenced by both the donor cell size and the relative densities of the parent (donor and recipient) cell numbers. Here the maximum number of transconjugants was obtained when the donor to recipient ratio was 54 : 1 and the lowest when the ratio was 0.07 : 1. The number of transconjugants was also highest when high population densities were used with a 3 log increase in transconjugants as the number of donor and recipient cells were increased from 10^7 to 10^8 cfu ml⁻¹, a 1 log increase in numbers. However, the natural population in the water phase rarely approaches these figures and, hence, plasmid transfer in water is likely to be a rare event. Sandt and Herson (1991) have further reported that high densities of parent organisms (of the order of 10^8 to 10^9 cfu ml⁻¹) are required to obtain triparental recombinants in drinking water. O'Morchoe *et al.*(1988), who examined conjugal transfer in laboratory and field studies, demonstrated that plasmids transferred *in vitro* were genetically and physically stable whereas a significant number of transconjugants recovered *in situ* contained deletions and genetic rearrangements. This suggests that

increased genetic instability may be a result of environmental stress imposed by biological or physical factors found in the fresh water environment.

Caldwell *et al.* (1989) showed, in their work to investigate the effect of starvation-survival on plasmid expression and maintenance in fresh water, that bacteria can regulate plasmid DNA and its products in several ways during starvation. First, if the plasmid is maintained intact, the products can either be present in an active form or be absent. Alternatively the host can degrade the plasmid, either partially or completely, as a source of energy or nucleic acids for biosynthesis to elevate cellular levels of RNA, as has been noted during starvation of a marine *Vibrio* sp. (Amy *et al.*, 1983b). This mechanism could prime the cell for rapid protein synthesis during recovery from starvation. Plasmid-borne resistances to antibiotics and/or heavy metals can provide a convenient system for examining plasmid and protein changes during long-term starvation-survival (Caldwell *et al.*, 1989).

In this chapter it has been shown that it is possible to transfer a plasmid between two disparate species of bacteria, *E. coli* and *A. hydrophila*, in a medium devoid of nutrients. The addition of a source of metabolisable nutrient, such as a carbon or amino acid source, or inorganic phosphate increased the efficiency with which the plasmid was transferred between these species. However no transfer was seen to occur in unamended water at temperatures of 15°C or below or at low population densities. This suggests that plasmid R1 *drd*-19 will be unlikely to be transferred from *E. coli* to *A. hydrophila* or vice versa in natural waters regardless of the organic or nutrient content of the water.

Chapter 7

Changes in Protein Composition of *Aeromonas hydrophila* under Starvation and Stress conditions

7.1 Introduction

Data obtained to date suggest that a multitude of adaptive metabolic and morphologic responses in aquatic bacteria take place as a result of an energy and nutrient downshift. The lack of exogenous energy and nutrients induced alterations of the bacterial cell surface characteristics and decreased endogenous respiration. Degradation of endogenous cell material, increased initial respiratory activity and rate of amino acid uptake and incorporation, an increased uptake capacity for glucose and mannose, a continuing synthesis of exoproteases, an increased adhesion to hydrophobic surfaces as well as alterations in cell constituents, e.g., membrane fatty acids, peptidoglycan constituents, and poly- β -hydroxybutyrate were observed (Dawson *et al.*, 1981; Kjelleberg & Hermansson, 1984; Marden *et al.*, 1985; Albertson *et al.*, 1990a, 1990c).

In addition the synthesis and degradation of proteins have been considered to be essential for survival of the bacteria during carbon and total energy and nutrient starvation in aquatic environments (Nystrom *et al.*, 1988; Albertson *et al.*, 1990b; Holmquist *et al.*, 1993). Protein synthesis is a dominant metabolic process in rapidly growing bacterial cells. The translocational machinery comprises about half the dry weight of the cell, and the process consumes up to 80% of the cell's energy (Maaloe, 1979). By that process amino acids are linked together by peptide bonds to form long linear polypeptides. The formation of a peptide bond itself is relatively simple in chemical terms: an activated carboxyl group of one amino acid condenses with an amino group of another amino acid. However proteins comprise up to 20 different amino acids arranged in precise sequences as determined by the genetic material of the cell. Protein synthesis therefore involves a complex apparatus which translates information encoded in nucleic acid sequences into amino acid sequences in proteins, and is a key component in the overall pathway of gene expression (Hershey, 1987).

A broad overview of protein synthesis follows. An amino acid is activated by formation of a high-energy ester bond to a specific tRNA in an ATP-consuming reaction catalyzed by an aminoacyl-tRNA synthetase. Aminoacyl-tRNAs are linearly ordered by interacting with a template, the mRNA; in effect, the tRNA molecule acts as an adapter between the amino acid and the mRNA. Such interactions occur on the surface of a cellular organelle called the ribosome. The polymerization process is divided conceptionally into three phases: initiation, elongation, and termination. During initiation, a unique initiator aminoacyl-tRNA binds at a precise region of the mRNA, thereby specifying the phase in which the mRNA is translated. Elongation is a cyclic process whereby subsequent aminoacyl-tRNA molecules bind to the ribosome as dictated by the mRNA. In each case, a peptide bond is formed by transfer of the aminoacyl (peptidyl) moiety to the amino group of the incoming aminoacyl-tRNA, and the ribosome progresses down the mRNA. During termination, the mRNA signals the binding of a factor that results in the hydrolysis of the completed peptidyl-tRNA. Polymerization on the ribosome is promoted by soluble protein factors specific for the three phases of protein synthesis. Thus the protein grows from its N terminus towards its C terminus, and the sequence of amino acids is colinear with the nucleic acid sequence in the mRNA. Energy is consumed during the charging of the amino acids to their tRNAs and during the polymerization process. More than 150 macromolecular elements are involved in the translational machinery (Hershey, 1987).

Intracellular hydrolysis of peptide bonds is important in both growing and starving bacterial cells. Defective or abnormal (structurally altered) polypeptides of various types are selectively degraded in growing cells, and extensive breakdown of normal protein occurs in nongrowing cells undergoing a variety of physiological stresses (Miller, 1987). The first inferences concerning protein degradation in *E. coli* were drawn from experiments designed by Rotman and Spiegelman (1954). Conditions that allowed detection of intracellular protein degradation in *E. coli* were

discovered by Mandelstam (1958, 1963), who found that, although he could detect little protein breakdown in growing cells (< 1% per hour), starvation for carbon, nitrogen, or amino acids led to an increased degradation rate (from about 1% to 5-6% per hour). These results indicated that the purpose of this enhanced proteolysis may be to provide the starving cell with a source of amino acids for the synthesis of postexponential phase proteins required for adaptation under starvation or shift-down conditions (Reeve *et al.*, 1984b).

Increased proteolysis during nutrient deprivation could result from synthesis of new proteases and/or activation of the preexisting enzymes (Matin *et al.*, 1989). The finding *in vitro* that protein substrates can allosterically increase protease activity (Waxman & Goldberg, 1986) suggests that activation of preexisting proteases is involved. Such activation could be caused by the abnormal proteins that stressed cells synthesize. Groat *et al.* (1986) showed that during the first 4 to 5 h of starvation for carbon substrates (glucose or succinate) approximately 30 proteins were induced in *E. coli* K-12 and that these proteins are important in starvation survival. Starvation-induced proteins have different temporal patterns of synthesis - some are synthesized very transiently during starvation, whereas others have a broader peak of synthesis (Groat *et al.*, 1986). Other investigators also showed that unique genes were switched on and new proteins were synthesized at the onset of starvation (Spector *et al.*, 1988). In addition Buettner *et al.* (1973) reported that intracellular cAMP (adenosine 3',5'-cyclic monophosphate) levels in *E. coli* increased at the onset of carbon starvation and there appeared to be an inverse relationship between the energetic state of the cell and cAMP levels. This raises the possibility that cAMP acts as a signal for starvation protein synthesis.

Nystrom and Kjelleberg (1989) demonstrated a starvation-related production of exoproteases, which appears to be positively regulated by the signal molecule cAMP. Actually Schultz *et al.* (1988) reported that about two-thirds of the starvation proteins in *E. coli* required a cAMP-cAMP receptor protein (CRP) complex for induction during starvation, but the other one-third is independent of

this control. They proposed the designation *cst* for cAMP-dependent carbon starvation response genes and *pex* for genes encoding the cAMP-independent starvation proteins. Most of the cAMP-dependent starvation proteins were induced only when the starvation regime involved deprivation of carbon, and it is likely that the increase in intracellular cAMP levels that occurs at the onset of carbon starvation plays a role in this induction. In contrast, nearly all the cAMP-independent proteins were induced regardless of whether the starvation state was carbon, nitrogen, or phosphorus deprivation.

Schultz *et al.* (1988) suggested that induction of the cAMP-dependent proteins was not involved in conferring resistance to starvation and these proteins were instead concerned primarily with preparing the cell for escape from starvation. It is known that many cAMP-controlled proteins mediate transport or catabolism of carbon substrates (Botsford, 1981). Thus induction of such proteins during starvation would be advantageous in nature, since it would enlarge the range of substrates that a bacterium can utilize without a lag, thus increasing its chances to escape starvation. An additional role for these proteins might be to facilitate bacterial adhesion to surface, since some starved bacteria demonstrate enhanced adherence capabilities (Kjelleberg, 1984). The work of Spector *et al.* (1988) reported that some cAMP-independent starvation proteins are important in starvation survival of *S. typhimurium*. It was further proposed that the proteins commonly induced by different nutrient deprivations were likely to be involved in conferring resistance to starvation (Groat *et al.*, 1986). That the induction of most of these proteins is independent of cAMP and CRP positive control is consistent with this notion, since the signal role of cAMP is confined to perturbations of the carbon and energetic state of the cell (Botsford, 1981).

Some starvation-inducible proteins are also induced by stresses such as a temperature upshift and nalidixic acid and exposure to cadmium ions (Nystrom *et al.*, 1990a, 1990b). Nalidixic acid has been reported to induce both the heat shock (*rpoH*-controlled) and SOS (*lexA*-controlled) regulons (Krueger & Walker, 1984),

while cadmium ions strongly induce the heat shock, SOS and oxidation stress responses (*oxyR*-controlled) in *E.coli* (Van Bogelen *et al.*, 1987). However, only a minor fraction of the proteins that are induced during either a temperature upshift or exposure to nalidixic acid or cadmium are synthesized in increasing amounts in response to multiple-nutrient starvation in *Vibrio* sp. strain S14 (Nystrom *et al.*, 1990b), suggesting that starvation does not provoke a full induction of any of the stress regulons described above, at least in *Vibrio* S14.

Although starvation for individual nutrients and heat shock, nalidixic acid, and cadmium treatment provoke unique and individual patterns of protein expression, some proteins were common to different starvation and stress treatments. However the proteins of one stimulon did not respond coordinately to all starvation and stress treatments, and relatively few of the starvation-inducible proteins were found to overlap with these induced by stress. This suggests, despite an interconnecting regulation for a few specific proteins, major differences in the regulatory pathways controlling the expression of starvation and different stress proteins. In addition, none of the proteins that overlapped between starvation and stress treatments were detected as being universal, nonspecific responders to stress. Thirteen of the starvation-inducible proteins induced by multiple starvation were unique and not observed when the cells were starved for any of the individual nutrients, indicating that additional sensors and signals may be involved when the bacterial cells are starved for several different nutrients simultaneously (Nystrom *et al.*, 1990b).

When the shift is to a higher temperature, the adaptation of mesophilic bacteria like *E.coli* involves at least two distinct control mechanisms. One mechanism is the heat shock response. The other mechanism is the control of growth rate by the availability of endogenous methionine, that is limited due to the extreme temperature sensitivity of the first enzyme in the pathway-homoserine transsuccinylase (HTS). It is considered that these two control mechanisms are linked, as HTS is also a heat shock protein (Ron *et al.*, 1990). The heat shock

response is a genetic response to environmental stress. Induction of the heat shock response in *E.coli* involves the expression of at least 17 genes, and is caused by stimuli, such as hydrogen peroxide or ethanol treatment, UV irradiation and viral infection (Neidhardt *et al.*, 1984), in addition to heat. When *E.coli* is exposed to a brief, sublethal dose of heat it becomes more resistant to subsequent, potentially lethal heat exposure. This phenomenon is termed thermotolerance and has been observed in many organisms (Lindquist, 1986). Oxidative stress can be functionally defined as an excess of peroxidants in the cell. Active oxygen species, such as superoxide anion and hydrogen peroxide, are produced as an inescapable by-product of normal aerobic metabolism, and their production is further enhanced by exposure to certain environments or by dietary or disease conditions. These active oxygen molecules can oxidize membrane fatty acids, thus initiating lipid peroxidation, can oxidize proteins, and can damage DNA and RNA. Oxygen toxicity results when the degree of oxidative stress exceeds the capacity of the cell defence systems. Enteric bacteria have several enzymes that may protect cells from oxidative damage, including superoxide dismutase and catalase (Farr & Kogoma, 1991).

7.2 Results

7.2.1 Protein fingerprint patterns of two-dimensional electrophoresis separations of whole cell extracts of *A. hydrophila* in lake water microcosms

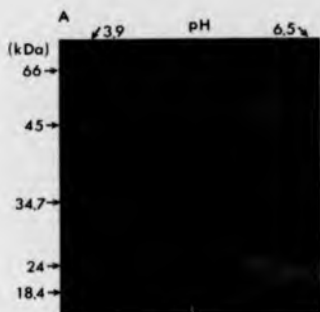
To determine what changes in proteins took place during the starvation process, two-dimensional acrylamide gel separations of the proteins from whole cells, starved in lake water at 4° to 37°C for up to 28d were analyzed. Standard polypeptide patterns at the onset of starvation are presented in Fig 7.1 A. Fig 7.1 B,C and Table 7.1 A revealed that at 4°C most of starvation-inducible proteins (75%) were synthesized in the early stages of starvation stress. 43 polypeptides were newly induced within 4 h of starvation. Among them 27 proteins remained throughout the entire starvation period whereas the rest (16 proteins) were synthesized only transiently, and were again absent within 28d. These data also showed that an additional 14 starvation-inducible proteins were found to be sequentially synthesized during the 28 d starvation period. In addition to the induction of new proteins during starvation, several protein spots were repressed after the commencement of starvation conditions. 4 polypeptides were repressed during 4h starvation stress and subsequently 37 proteins disappeared after a 28 d starvation period. A similar pattern of results was observed in starved cells at temperatures from 15° to 37°C. At 15°C, 32 starvation-related proteins were induced during the 4h starvation period including 2 proteins which were transiently induced. These were subsequently repressed after day 3. In addition 32 proteins were sequentially synthesized during the 7d starvation period. 22 proteins were found to disappear from the initial protein fingerprints after 7d of starvation stress (Fig 7.1 D,E,F, Table 7.1B). 37 starvation-inducible proteins were newly synthesized and 2 proteins disappeared within 4h under starvation conditions at 25°C. At the optimal growth temperature, 30°C, 55 polypeptides were induced during 4h starvation and 12

Fig 7.1 Protein fingerprint of two-dimensional electrophoresis separations of whole-cell extracts of *A. hydrophila* starved in lake water microcosms

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. These were incubated in the dark.

	Incubation temperature	Starvation period
A	Stationary phase cells	
B	4°C	4 hr
C	4°C	28 d
D	15°C	4 hr
E	15°C	3 d
F	15°C	7 d
G	25°C	4 hr
H	30°C	7 d
I	30°C	28 d
J	37°C	3 d
K	37°C	7 d

- Starvation-induced proteins
- △ Location of proteins repressed by starvation
- ◐ Transiently induced starvation proteins
- ◑ Location of proteins repressed after transient induction
- Commonly induced-proteins at 4°, 15° and 25°C during 4 hr starvation



D



E



F



G



H



I



J



K

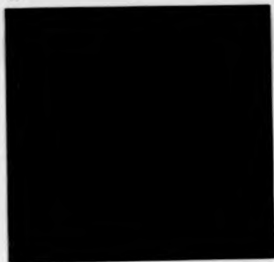


Table 7.1 Changes in the protein fingerprint of whole cell extracts of *A. hydrophila* starved in lake water microcosms at different temperatures

A. Incubation temperature ; 4°C

Starvation period		Numbers of protein
4 hr	28 d	
+	+	27
+	-	16
	+	14
		4
	-	37

Key + : Induction , - : Repression

B. Incubation temperature ; 15°C

Starvation period			Numbers of protein
4 hr	3 d	7 d	
+	+	+	30
+	-	-	2
	+	+	10
	+	-	7
		+	15
			4
	-		11
		-	7

Key + : Induction , - : Repression

C. Incubation temperature ; 25°C

Starvation period	Numbers of protein
4 hr	
+	37
-	2

Key + : Induction , - : Repression

(continued on next page)

Table 7.1 - continued

D. Incubation temperature ; 30°C

Starvation period		Numbers of protein
7 d	28 d	
+	+	43
+	-	12
	+	16
		16
	-	14

Key + ; Induction - ; Repression

E. Incubation temperature ; 37°C

Starvation period		Numbers of protein
3 d	7 d	
+	+	29
+	-	4
	+	6
-		7
	-	16

Key + ; Induction - ; Repression

F. The comparison of protein numbers induced at different temperatures during 4 h starvation period

Incubation temperature	Total proteins induced	Temperature-specific proteins induced	Commonly induced proteins at three temperatures
4°C	43	13	30
15°C	32	2	
25°C	37	7	

of these were identified as proteins induced only transiently during starvation. Subsequently a further 16 inducible proteins were identified by day 28. 30 proteins from the initial protein fingerprint were repressed sequentially during the 28 d starvation period. 33 starvation proteins were induced at 37°C in a starvation period. 29 of these were still present at day 7 but 4 of the initial proteins induced were repressed after day 3. An additional 6 starvation-inducible proteins were present by day 7 of the starvation period. Repression of 7 polypeptides took place over the first 3 days and another 16 proteins subsequently disappeared during a 7 d starvation period (Fig 7.1G, H, I, J, K, Table 7.1C, D, E).

7.2.2 Densitometric measurements of the intensity of proteins produced by *A. hydrophila*

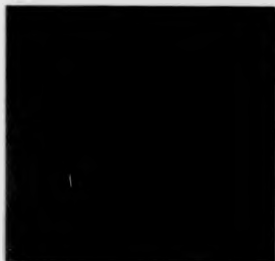
Several protein spots from two-dimensional electrophoresis separations exhibited increased or diminished intensity during the process of starvation stress. Therefore protein intensities on a few representative protein spots were examined after 7 day of starvation stress at different temperatures. Fig 7.2A and Table 7.2 display the initial densitometric volume of protein spots A, B, C, D, and E. The intensity of proteins A and B was decreased during the 7 d starvation stress period compared to the control, and this decrease was found to be temperature-dependent, with the intensity of both protein A and B decreasing simultaneously with an increase in temperature. In contrast densitometric readings of proteins C, D and E were increased by 3.0 to 15.1 times compared to the control after 7 d starvation. The intensities of these three spots after 7 d of starvation stress showed higher values at the low temperature, 15°C, and at the submaximal temperature, 37°C, compared with that at optimal temperature, 30°C. Proteins C, D, and E could be more closely related to low and high temperature stress rather than to starvation stress (Fig 7.2 B, C, D, Table 7.2).

Fig 7.2 Densitometric measurements on protein intensity of *A.hydrophila*

A.hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. All flasks were incubated in the dark. Changes in the intensity of proteins (A, B, C, D, E) were examined by densitometry after 7d-incubation at different temperatures.

	Incubation temperature	Starvation period
A	Stationary phase cells	
B	15°C	7 d
C	30°C	7 d
D	37°C	7 d

A



B



C



D



Table 7.2 Changes in protein intensity of whole cell extracts of *A.hydrophila* at different temperatures during 7 day-starvation

● Protein intensity

Protein (Fig 7.2)	Control (Day 0)	Densitometric volume		
		15°C (7 d)	30°C (7d)	37°C (7d)
A	1288	973	720	303
B	1612	532	460	428
C	65	451	196	716
D	112	1048	830	1688
E	192	1546	287	1963

7.2.3 The changes in protein fingerprint pattern of whole cell extracts of *A. hydrophila* with nutrient amendments in lake water microcosms

To examine whether other proteins different from starvation-inducible proteins could be induced by incubation in a starvation medium in the presence of different nutrients, such as glucose, serine, casein and phosphate, all were added to nutrient-amended lake water. Fig 7.3 A-F and Table 7.3 showed that many different polypeptides were newly synthesized and repressed during a 7 d starvation period after the addition of carbon, nitrogen, and phosphorus sources to lake water compared with those in the unamended control. Further subsequent changes in proteins were observed during a 28 d starvation period.

7.2.4 Stress-induced proteins in whole cell extracts of *A. hydrophila*

Several treatments inducing stress in cells were used to investigate the occurrence of stress-inducible proteins. After heat shock at 58°C, 24 polypeptides were induced within 10 min, and an additional 4 and 26 heat-inducible protein spots were sequentially synthesized after 20 min and 1 h respectively. 19 proteins were found to have disappeared during the first 1 h of heat stress. Protein a was continuously intensified throughout the entire period of heat treatment. Proteins b, c, d and e were increased in intensity between 20 min and 1 h (Fig 7.4A, B, C, Table 7.4). In addition protein f was found to be induced transiently only under heat stress for and then repressed (Fig 7.4B, C).

When cells were treated with 10% ethanol(v/v) and 10 mM cadmium chloride, 36 ethanol-induced and 35 cadmium-induced protein spots were observed. 23 and 16 proteins were repressed under ethanol and cadmium stress respectively (Fig 7.4D, E, Table 7.4). Fig 7.4A-E and Table 7.4 reveal that many common proteins were induced under the various stress-conditions. Proteins b, c, d, m, n, and q were synthesized simultaneously when the cells were

Fig 7.3 The changes in protein fingerprint of whole cell extracts of *A. hydrophila* with nutrient amendments in lake water microcosms

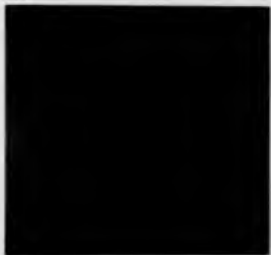
A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms. Different nutrient sources were added to the flask and the flasks were incubated in the dark at 30°C.

	Nutrients added	Incubation period
Fig 7.1H	None (control)	7 d
A	D-glucose (0.5g-C/l)	7 d
B	L-serine (100mg/l)	7 d
C	Casein (100mg/l)	7 d
D	Casein (100mg/l)	28 d
E	PO ₄ (100mg/l)	7 d
F	PO ₄ (100mg/l)	28 d

□ Induced proteins

△ Location of repressed proteins

A



B



C



D



E



F



Table 7.3 Changes in protein fingerprint pattern of whole cell extracts of *A. hydrophila* with nutrient amendments

Nutrient sources amended	Concentration	Incubation period at 30°C			
		7 d		28 d	
		+	-	+	-
None		0	0	0	0
D-glucose	0.5 g-C/l	13	18	ND	ND
L-serine	100 mg/l	18	17	ND	ND
Casein	100 mg/l	26	13	11	19
PO ₄	100 mg/l	10	13	17	11

Key

- + : Protein numbers induced
- : Protein numbers repressed
- ND : Not determined

Fig 7.4 Stress-induced proteins of whole cell extracts of *A. hydrophila*

A. hydrophila was grown in nutrient broth at 30°C then transferred to filtered -autoclaved lake water microcosms. These were incubated in the dark.

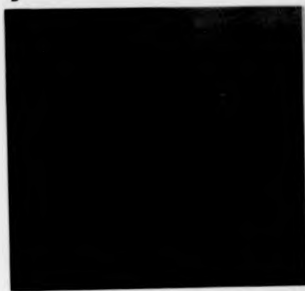
	Stress condition treated	concentration	Incubation temperature	Incubation time
A	Heat		58°C	10 min
B	Heat		58°C	20 min
C	Heat		58°C	1 hr
D	Ethanol	10 % (v/v)	30°C	1 hr
E	CdCl ₂	10 mM	30°C	1 hr
F	NaCl	5 % (w/v)	30°C	5 d
G	NaCl	7 % (w/v)	30°C	2 d
H	Nutrient broth containing 3.5 % NaCl (w/v)-grown cells			

- ☐ Stress-induced proteins
☐ Location of proteins repressed by stresses

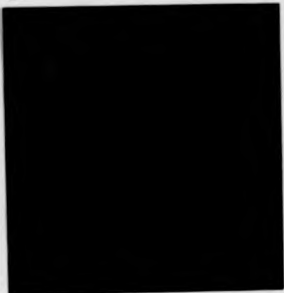
A



B



C



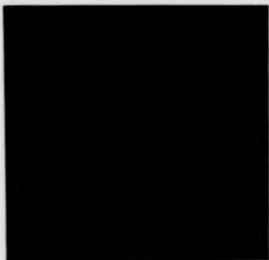
D



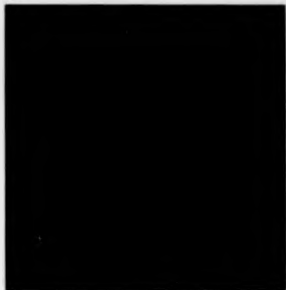
E



F



G



H



Table 7.4 Stress-induced proteins of whole cell extracts of *A. hydrophila*

1. Heat shock (58°C)

	10 min	20 min	1 hr	Total
Protein numbers induced	24	4	26	54
protein numbers repressed	6	6	7	19

• **Gradual increase in protein intensity by heat shock**

Protein a	Fig 7.3 A → 7.3 B → 7.3 C (10 min) (20 min) (1 hr)
Protein b, c, d, e	Fig 7.3 B → 7.3 C (20 min) (1 hr)

2. Ethanol shock (10 % v/v, 1 hr)

Protein numbers induced	36
Protein numbers repressed	23

3. Cadmium chloride stress (10 mM, 30 min)

Protein numbers induced	35
Protein numbers repressed	16

4. Commonly induced-proteins between two or three stresses

Ht	: Heat shock (1 hr)
Et	: Ethanol shock
Cd	: Cadmium chloride stress

Ht , Cd	6 proteins (Fig C , E ; b, c, d, m, n, q)
Et , Cd	2 proteins (Fig D , E ; r, s)
Ht , Et , Cd	5 proteins (Fig C , D, E ; a, g, h, o, p)

(continued on next page)

Table 7.4 - continued

5. Osmotic shock (30°C)

F	: 5 % NaCl (for 5 d)
G	: 7 % NaCl (for 2 d)
H	: Nutrient + 3.5 % NaCl grown cells

	F	G	H
Protein numbers induced	20	19	25
Protein numbers repressed	10	11	11

• Commonly induced-proteins from osmotic shock

F, G	2 proteins (Fig F, G; n, m)
F, H	3 proteins (Fig F, H; b, e, g)
G, H	2 proteins (Fig G, H; a, f)
F, G, H	2 proteins (Fig F, G, H; c, d)

subject to heat and cadmium shock (Fig 7.4C, E). Proteins r and s were both produced under ethanol and cadmium shock (Fig 7.4C, E). In particular proteins a, g, h, o and p were identified as being commonly induced under these three conditions (Fig 7.4C, D, E).

Osmotic stress induced a number of proteins dependent upon the osmotic stress conditions used, such as different sodium chloride concentrations and storage period (Fig 7.4F, G and Table 7.4). 20 and 19 polypeptides were newly synthesized and 10 and 11 proteins were repressed under osmotic stress imposed by 5% sodium chloride for 5 d, and 7% sodium chloride for 2 d respectively. Fig 7.4H and Table 7.4 show that several proteins were also induced by the cells grown in nutrient broth containing 3.5% sodium chloride. 25 proteins were induced by osmotic stress and 21 proteins were repressed. Moreover various proteins were shown to be synthesized simultaneously under different osmotic conditions. Proteins n and m were commonly produced under the stress imposed by 5% sodium chloride for 5 d and 7% sodium chloride for 2 d. Proteins b, e, and g were induced in both the starvation medium containing 5% sodium chloride and nutrient broth containing 3.5% sodium chloride, and proteins a and f, in both the starvation medium containing 7% sodium chloride and nutrient broth containing 3.5% sodium chloride. Proteins c and d were found to be synthesized under any of these three osmotic stress conditions (Fig 7.4F, G, H, Table 7.4).

7.2.5 Protein fingerprint patterns of two-dimensional electrophoresis separations of periplasmic proteins of *A. hydrophila* starved in lake water microcosms

To examine changes in periplasmic proteins of the cells in response to starvation stress, *A. hydrophila* was incubated in lake water at different

Table 7.5 Changes in periplasmic protein fingerprint pattern of *A. hydrophila* starved in lake water microcosms

- Starvation period ; 2 weeks

	4°C	15°C	30°C
Protein numbers induced	4	9	9
Protein numbers repressed	24	36	24

temperatures for 2 weeks. Table 7.5 shows that several different periplasmic proteins were induced and repressed in a temperature-dependent fashion under starvation conditions. Four, nine and nine proteins were induced and 24, 36, and 24 proteins were repressed at 4°, 15° and 30°C respectively during the 2 week starvation period. Only one protein was identified as being induced under starvation stress at all temperatures from 4° to 30°C.

7.2.6 Stress-induced periplasmic proteins of *A. hydrophila*

The effect of other stresses on changes in periplasmic proteins of *A. hydrophila* was also studied. Fig 7.5A and Table 7.6A show that many proteins were found to be increased in intensity after the imposition of ethanol stress for 30 min to the cells. One protein, termed L1, was intensified by 4.5 times in densitometric measurements over the control. This protein was identified as a protein intensified transiently only after ethanol stress for 30 min, and was repressed again after ethanol stress for 2 h. Table 7.6B shows the terminal sequence of the ethanol stress-induced protein L1.

When heat shock was imposed on the cells, protein G1 was shown to be increased by densitometric measurements by 8.3 times after 30 min-heat shock (Fig 7.5B, Table 7.6C). Table 7.6D shows the N-terminal sequence of the heat shock-induced protein G1. As a consequence of employing other stresses, such as hydrogen peroxide, cadmium chloride and osmotic shock to the cells, 22, 1 and 9 periplasmic proteins were induced and 22, 4 and 14 proteins were repressed respectively. Some proteins also increased in density to some degree after hydrogen peroxide and cadmium chloride stress (Table 7.6E).

Fig 7.5 Microsequencing blot for identification of N-terminal sequence of stress-induced proteins

A. hydrophila, grown in nutrient broth at 30°C then transferred to filtered-autoclaved lake water microcosms, was incubated in the dark under the stress of ethanol and heat treatment as follows;

Figure No.	Stress	Concentration	Incubation temperature	Incubation time
A	Ethanol	10% (v/v)	30°C	30 min
B	Heat		58°C	30 min

After 2-D gel running and microsequencing blot, N-terminal sequence on stress-induced proteins was analysed.

Fig. A : ethanol-shock protein L1

Fig. B : heat-shock protein G1

A



B



Table 7.6 Stress-induced periplasmic proteins of *A. hydrophila*

A. Changes in protein intensity by ethanol shock

Protein No.	Densitometric volume	
	Control	Ethanol shocked (Fig 7.5 A)
L1	1011	4520

B. N-terminal sequence of ethanol shock-induced protein L1 (Fig 7.5 A)

No.	Residue	Amount (p moles)
1	Alanine	9.09
2	Phenylalanine	6.82
3	Glutamic acid	6.15
4	Leucine	5.92
5	Proline	4.00
6	Alanine	7.27
7	Leucine	11.84
8	Proline	8.00
9	Tyrosine	2.00
10	Alanine	9.55

(continued on next page)

Table 7.6 - continued

C. Changes in protein intensity by heat shock

Protein No.	Densitometric volume	
	Control	Heat shocked (Fig 7.5 B)
G1	485	4040

D. N-terminal sequence of heat shock-induced protein G1 (Fig 7.5 B)

No.	Residue	Amount (p moles)
1	Serine	3.12
2	Alanine	23.72
3	Glutamine	10.01
4	Asparagine	7.54
5	Methionine	6.68
6	Leucine	10.83
7	Alanine	15.05
8	Leucine	18.02
9	Isoleucine	8.01
10	Glutamine	7.92
11	Glutamic acid	4.62
12	Serine	3.00
13	Glutamic acid	5.38
14	Valine	6.67

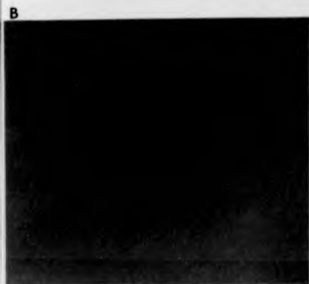
E. Stress-induced periplasmic proteins of *A. hydrophila*

	H ₂ O ₂ (10 %, v/v)	CdCl ₂ (10 mM)	NaCl (5% for 5 d)
Protein numbers induced	22	1	9
Protein numbers repressed	22	4	14

Fig 7.6 Identification of alkaline phosphatase by two-dimensional gel electrophoresis in the proteins of phosphate-limited *A. hydrophila*

- A. Standard two-dimensional electrophoresis gel of the protein of *A. hydrophila* grown in phosphate-limited minimal medium.
- B. Identification of alkaline phosphatase using α -naphthyl- PO_4 .
- C. Microsequencing blot.
- D. Two-dimensional gel obtained from the proteins of phosphate-limited minimal medium grown *A. hydrophila* cells incubated in minimal medium deprived of carbon, nitrogen and phosphorus sources at 30°C during 14 d.

► ; indicates alkaline phosphatase



7.2.7 Identification of alkaline phosphatase of *A. hydrophila*

This experiment shows the location of the alkaline phosphatase of *A. hydrophila* on the two-dimensional gel. Fig 7.6A shows two-dimensional electrophoresis separations of the proteins of *A. hydrophila*, which has been grown in phosphate limited-minimal medium (2 mg PO_4/l). This figure shows the exact location and approximate molecular weight (Ca. 55 kDa) of alkaline phosphatase of *A. hydrophila*. Fig 7.6B shows alkaline phosphatase (brown spot) of *A. hydrophila* which was identified by the use of the colorimetric reagent, α -naphthyl- PO_4 . Fig 7.6C presents a microsequencing blot, which was conducted to identify the N-terminal sequence of alkaline phosphatase of *A. hydrophila* but failed to do so because the optimal amount of protein necessary for analyzing N-terminal sequence was not retained on the blot paper. Fig 7.6D also reveals the distinct spot of alkaline phosphatase of *A. hydrophila* which is shown to be produced clearly after the deprivation of carbon, nitrogen, and phosphorus sources for 14 d.

7.3 Discussion

The results presented in this study demonstrated that many starvation-specific proteins were induced in the first 4 hr after the onset of starvation stress at temperatures between 4° and 25°C. Many of these starvation proteins were commonly induced under a wide range of temperatures from 4°C to 25°C (near optimum temperature). Some proteins also were synthesized at specific temperatures under starvation conditions, with the induction of more proteins occurring at 4°C than 15° and 25°C. These data indicate that cells appear to rearrange intracellular proteins at an early stage in response to starvation stress and that protein synthesis during the initial phase of starvation is likely to be essential for conferring starvation resistance. The common proteins probably play a significant role in the survival of *A. hydrophila* under starvation conditions regardless of the temperatures imposed. The specific proteins may be necessary only at specific temperatures for the survival of cells under starvation conditions.

Lemaux *et al.* (1978) reported that shifts up or down in growth temperature of even a minor nature were accompanied within 1 or 2 min by large changes in the differential rates of synthesis of many *E. coli* proteins. There is a gradient of response from some proteins that are hyperinduced transiently 100-fold to some that for a time virtually cease to be synthesized. In general, the greater the shift in temperature the larger the response but transient inductions and repressions are easily detected even for shifts of a few degrees within the normal range of growth temperatures. Proteins induced by a shift from one temperature to a higher one are repressed upon return to the lower temperature, and vice versa. Within the normal temperature range these transient changes quickly fade, and in 15 to 20 min the synthesis rates return to the preshift values for most proteins (Lemaux *et al.*, 1978). In effect, little permanence seems to be achieved, suggesting that the transient inductions and repressions are passive responses to temperature shifts and that they are counteracted by appropriate

adjustments of gene activity. However for a few proteins temperature-shifted within the normal range and for a large number (>100) of proteins temperature-shifted to the high range, the transient inductions and repressions rapidly adjust cellular content to new steady-state levels, higher or lower than at the preshift temperature. The heat shock proteins are found among those that are hyperinduced upon a shift to high temperatures. The analysis of the effect of growth temperature on the cellular content of 133 proteins (constituting >70% of the protein mass of the cell) revealed that, except for a few proteins that vary in level across the entire range of growth temperature, cellular proteins do not change appreciably within the normal range of growth temperature. Electrophoresis gels of protein produced by *E. coli* cells grown at 23°C and at 37°C were indistinguishable to the untrained eye (Herendeen *et al.*, 1979). In contrast the patterns at 13° or 46°C were quite distinctive. Many proteins were increased or reduced in amount (up to 25-fold) during steady-state growth in the high or low range of growth temperature; a few were not easily seen on autoradiograms except at extreme temperatures.

In general, proteins involved in transcription or translation (and under stringent control) are present in reduced levels during restricted growth at high and low temperatures, and some proteins known to be involved in energy metabolism display the opposite response and are greatly increased in level in both high and low ranges (Herendeen *et al.*, 1979). Jones *et al.* (1987) showed that *E. coli* cells responded to a shift to a low temperature by inducing a set of proteins including *nusA*, initiation factor 2 α , initiation factor 2 β , and polynucleotide phosphorylase, which are involved in transcription, translation and possibly mRNA degradation. Although *nusA* is involved in both termination and antitermination of transcription, its primary function is believed to be transcription termination. Initiation factor 2 is responsible for mediating binding of charged tRNA to the small ribosomal subunit preparatory to initiation of

translation. Polynucleotide phosphorylase may function as an mRNA RNase in cells.

It has been demonstrated, in this study, that starvation-specific proteins were sequentially induced time-dependently during the whole period of starvation at all temperatures from 4° to 37°C, and that some proteins showed transient induction after a specific time of starvation followed by repression. Throughout the experimental period several proteins were sequentially repressed when cells were subject to starvation conditions. In addition the data showed that the intensity of proteins analysed was affected under starvation stress in a temperature dependent fashion. These results suggest that those changes such as sequential induction and repression, and intensification in individual proteins are likely to be a process of adaptation of the cells for survival.

The fact that some of the proteins induced in *Vibrio* sp. during nutrient and energy deprivation may be involved in substrate capture and transport was reported by Marden *et al.* (1987), who showed that two separate binding protein-dependent uptake systems for amino acids were modulated differently during starvation. The V_{max} of the high-affinity uptake system increased during starvation, while the opposite occurred for the low-affinity system. Another aspect of substrate capture during starvation is indicated by the production of extracellular proteases by *Vibrio* sp. Proteases capable of degrading a wide range of substrates were exuded even after cells were starved for 120 h.

Nystrom *et al.* (1990b) described the response of a marine *Vibrio* sp. strain S14 to multiple-nutrient starvation (i.e., starvation for glucose, amino acids, ammonium and phosphate simultaneously) as a three-phase process based on the kinetics of macromolecular synthesis and the reorganization of macromolecular contents. The first phase, defined as the stringent control phase, encompasses an accumulation of guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and decreases in the rate of RNA and protein (Nystrom *et al.*, 1990b), and peptidoglycan (Nystrom & Kjelleberg, 1989) synthesis. A pronounced increase

in the rate of proteolysis during this phase was demonstrated for *Vibrio* S 14 cells (Nystrom *et al.*, 1988) during the first 40 min. In the second phase, there was a temporary increase in respiratory activity (Marden *et al.*, 1985), of RNA and protein synthesis (Nystrom *et al.*, 1990b), and peptidoglycan synthesis (Nystrom & Kjelleberg, 1989) between 1 and 3 h in parallel with the diminishing pool of ppGpp. The third phase included a gradual decline in macromolecular synthesis after 3 h.

Two-dimensional gel electrophoresis of pulse-labeled proteins revealed that a total of 66 proteins were identified as starvation inducible, temporally expressed throughout the three phases of starvation, and that the early temporal class of starvation proteins, which included the majority of the starvation inducible proteins, was the most essential for long-term survival. In addition it was demonstrated that *Vibrio* sp. strain S 14 cultures prestarved for 1 h for glucose, amino acids, ammonium, or phosphate as well as cultures exposed for 1 h to cadmium chloride exhibited longer survival during the subsequent multiple-nutrient starvation in the presence of chloramphenicol or rifampin. An amino acid starvation and cadmium chloride exposure, which induced the stringent response, were the most effective in conferring enhanced survival (Nystrom *et al.*, 1990b).

Reeve *et al.* (1984b) reported that protein synthesis was important for the survival of carbon-starved *E. coli* K 12. The addition of chloramphenicol to *E. coli* cells starved for carbon inhibited protein synthesis and markedly increased the death rate during starvation. Studies with *relA* mutants indicated that the deleterious effect of chloramphenicol on the starvation survival was not mediated through interference with the stringent response but rather was due to protein synthesis inhibition. Cells starved in the presence of amino acid analogues also exhibited decreased survival. The survival rate during starvation correlated with the rate of protein breakdown which, by making free amino acids available, in turn determined the rate of protein synthesis (Reeve *et al.*, 1984a). Willetts

(1967) demonstrated the presence of a pool of labile proteins in logarithmically growing *E. coli*, and it is possible that some of the protein synthesis occurring at the onset of starvation serves to replenish these rapidly degraded proteins. Jouper-Jaan *et al.* (1986) who studied new protein synthesis during starvation survival of marine isolates, reported that the possibility that the appearance of new protein spots was a result of degradation, which indicates the degradation of certain proteins in the cells furnish the energy and building blocks for the synthesis of other proteins, may not be ruled out, and also suggested that the formation of large amounts of other protein, however, was the result of *de novo* synthesis during starvation.

Starving cells very likely synthesize proteins whose purpose is to increase the probability of escaping starvation. It is well-established that organisms subjected to growth under carbon limitation depress the synthesis of catabolic enzymes, thus amplifying their potential to make use of diverse carbon substrates and thereby escaping carbon limitation (Matin *et al.*, 1976). However, the essential biosynthetic activity at the onset of starvation appears to be concerned with the synthesis of proteins which help the bacterium to withstand the starvation stress *per se*, rather than to escape it. A cell entering starvation faces a fundamentally altered physiological situation. The cell metabolism, formerly geared to growth, must be reoriented to maintenance. Endogenous energy reserves must be mobilized, and the cell must survive in the absence of multiplication. These changes probably require marked physiological and structural changes in the cells, and the proteins synthesized at the onset of starvation might help to achieve this change (Reeve *et al.*, 1984b).

Starvation proteins synthesized by *E. coli* at the onset of carbon starvation exhibited four temporal classes of synthesis in response to glucose or succinate starvation, indicating sequential expression of carbon starvation response (*cst*) genes. Further at least one *cst* mutant of *E. coli* was markedly compromised in starvation survival (Groat *et al.*, 1986). Both of these findings are reminiscent

of the process of sporulation in *Bacillus* spp. which, through the temporally ordered expression of unique genes, leads to the generation of resistant structures (Losick & Youngman, 1984). It is probable that non-spore-forming bacteria, such as *E. coli*, undergo a process of significant molecular realignment in response to starvation, which increases their resistance to this stress, and which may be akin to sporulation (Groat *et al.*, 1986).

The proteins that were synthesized transiently very early in starvation might be protease or peptidase specific to the starvation state (Groat *et al.*, 1986). *B. subtilis* produces proteases during the end of the log phase of growth and during the early stages of sporulation (Doi, 1972). In addition, an amino peptidase was found in *E. coli* whose levels were increased 4 fold during phosphate starvation (Lazdunki *et al.*, 1975). These proteases may possess altered specificity compared with those produced during active growth and may be involved in the degradation of growth-associated proteins at the onset of starvation. It is also conceivable that some of these early proteins are unique σ factors involved in the transcription of the starvation response genes. Unique σ factors direct the transcription of genes activated during the stationary phase in *B. subtilis* (Trempey *et al.*, 1985).

Nystrom *et al.* (1992) have suggested a number of observations from the visual inspection of the two-dimensional autoradiograms of the mesophilic *Vibrio* sp. strain S14. First, carbon depletion does not seem to be the major determinant for protein expression during multiple starvation. The carbon starvation stimulon did not overlap with the multiple-nutrient starvation stimulon to a greater extent than did the nitrogen and phosphorus starvation stimulons. Second, a fraction of the proteins belonging to the carbon, nitrogen, and phosphorus starvation stimulons was induced regardless of the starvation conditions encountered by the cells. This fraction was tentatively termed general starvation proteins. A similar overlap between starvation stimulons has previously been described for *E. coli* (Groat *et al.*, 1986), *S. typhimurium*

(Spector *et al.*, 1986) and *B. subtilis* (Hecker *et al.*, 1989). Third, four of the five starvation-specific proteins were not induced during simultaneous starvation for phosphate, nitrogen, and carbon. Finally a relatively large number of proteins (termed multiple-nutrient starvation-specific proteins) were induced exclusively in response to multiple-nutrient starvation and could not be grouped into any of the individual nutrient starvation stimulons. This suggests that the multiple-nutrient starvation stimulon is not merely the sum of individual-nutrient starvation stimulons, and that additional sensors and signals are involved in the response to simultaneous starvation for carbon, nitrogen, and phosphorus.

Long-term survival, dwarfing, and stress resistance were observed only for carbon or multiple nutrient-starved *Vibrio* strain S14 cells, while long-term nitrogen or phosphorus starvation in the presence of carbon resulted in significant cell death and only weak or transient development of stress resistance. These results indicate that the general starvation proteins of *Vibrio* sp. strain S14 are not directly involved in starvation survival or that these proteins are persistently synthesized only during long-term carbon or multiple-nutrient starvation (Nystrom *et al.*, 1992). In addition, carbon and multiple-nutrient starvation were the only conditions that provoked a rapid shutdown of RNA and protein synthesis during the initial phase of starvation and subsequently altered the rate of protein synthesis in a starvation phase-specific manner (Nystrom *et al.*, 1990a ; 1990b).

Spector *et al.* (1988) constructed a series of Mu d-directed *lacZ* operon fusion mutants for the study of starvation-regulated gene expression in *S. typhimurium*. A number of such Mu d *lac* insertions in starvation-inducible genes of *S. typhimurium* were found to impair the cells' ability to survive prolonged periods in the stationary phase. Furthermore, Tormo *et al.* (1990) isolated an *E. coli* strain with a mutation in a gene (*surA*) which was found to be essential for survival in stationary phase but not required for exponential growth. Similarly, the *kaiF* gene product suggested to serve as the α factor for a

starvation stress resistance regulon in *E.coli* was found to be not only required for thermotolerance and oxidative stress resistance but also essential for survival during prolonged periods of starvation in the absence of secondary stress (Lange & Hengge-Aronis, 1991 ; McCann *et al.*, 1991).

In studies investigating synthesis of membrane and periplasmic proteins of *Vibrio* sp. during starvation, Nystrom *et al.* (1988) showed that several outer and cytoplasmic membrane proteins, and periplasmic proteins increased throughout the starvation period, although the time at which they were induced ranged from 0 to more than 24 h after the onset of starvation. It is possible that several regulons are involved in a sequential starvation response of the cells. These may be induced by exogenous as well as a series of time-dependent endogenous energy and nutrient shifts. The function of protein degradation for the survival of starved bacteria has been described in several studies. Carbon-starved cells may need protein degradation to inactivate energy-wasting enzymes deleterious to them. It was shown that starving cells degraded their intracellular proteins at widely varied rates (Inloes *et al.*, 1983), suggesting that it is advantageous to those cells to selectively degrade certain proteins.

A number of enzyme activities involved in energy metabolism are known to be lost during starvation ; this loss may be due to proteolysis (Nystrom *et al.*, 1988). Miller (1987) suggested that the increased rate of proteolysis may be due to the fact that certain enzymes become more susceptible to degradation when their substrates are present in decreasing amounts during starvation. Reeve *et al.* (1984a) demonstrated that peptidase activities were important to the survival of *E.coli* and *S.typhimurium* cells during carbon starvation, and the more rapid loss of viability of carbon-starved mutants that were deficient in protein degradation was observed in comparison to the wild type. Yen *et al.* (1980) showed that the lack of four peptidases, N, A, B and D, resulted in a build up of small, TCA-soluble peptides in peptidase-deficient *S.typhimurium* mutants during carbon starvation. Some small peptides were found to inhibit the growth of organisms

that were unable to hydrolyse them (Miller, 1975). On the other hand, it appeared most probable that the decreased stability of the peptidase mutants in resistance to starvation resulted not from any toxic effects, but rather from the inability of these mutants to degrade protein (Reeve *et al.*, 1984a). Yen *et al.* (1980) showed that these peptidases played an important role in recovery of cells from nutritional shift-down. Thus, the peptidases are important to the survival of cells during nutrient limitation or deprivation, both conditions that normally result in enhanced rates of protein degradation. In peptidase-deficient mutants of *E. coli* starved for carbon, the rate of hydrolysis of TCA-insoluble polypeptide was much lower than that observed in the wild type (Reeve *et al.*, 1984a). This work is consistent with that of Yen *et al.* (1980), who reported that loss of peptidases in the cells resulted in a lower rate of protein degradation during starvation, and further that the products of protein degradation were small peptides, as opposed to the free amino acids generated by the wild type.

The results obtained in this study showed that the addition of nutrients, such as carbon, nitrogen and phosphorus source induced changes of proteins, and also showed the occurrence of sequential induction during the entire period of nutrient-amended experiments, suggesting that proteins, different from starvation-inducible proteins are sequentially expressed when nutrient amendments are added to the starvation medium. The study of Albertson *et al.* (1990b), who examined the kinetics of macromolecule synthesis in response to nutritional upshifts at different times of starvation, showed that while the rate of RNA synthesis was effectively inhibited in the presence of rifampicin, the relative rate of protein synthesis after the upshift of nutrition increased markedly even in the presence of rifampicin. This indicates that *de novo* RNA synthesis was not required for the initial rapid increase in protein synthesis and that rRNA and ribosomes may exist in excess. It was previously demonstrated that the proportionality between the number of ribosomes and the demands for protein synthesis broke down during slow growth of *E. coli* resulting in an excess of the

protein synthesizing machinery (Koch, 1979). He suggested that the extratable RNA in slowly growing carbon-limited *E. coli* was not involved in making proteins but may significantly increase the ability of the cells to respond rapidly to sporadic inputs of nutrients. Albertson *et al.* (1990d) found similar results concerning protein synthesis as a result of a nutritional upshift when *Vibrio* sp. S14 cells had been in a non-growing starved state for 24, 120 and 200 h. Possible changes in mRNA stability may not be involved in this response, since the starvation-induced stabilization of the mRNA pool is rapidly lost during the nutritional upshift.

Two-dimensional gel electrophoresis was used to demonstrate the fate of starvation-specific proteins during the nutritional upshift and the synthesis of recovery-induced proteins that were not found in starving cells. Most starvation-inducible proteins were repressed immediately at the onset of the nutritional upshift, while 21 proteins were found to exhibit novel or markedly increased expression as a consequence of substrate addition. 10 of these proteins were expressed both through the maturation phase (lag period) and during regrowth. However, 11 recovery-induced proteins identified were expressed exclusively during the maturation phase, and were subsequently repressed at the onset of regrowth (Albertson *et al.*, 1990b).

The possible role of such maturation-specific proteins related to reorganization of both the non-replicative nucleoid and the autolysis-resistant peptidoglycan (Nystrom & Kjelleberg, 1989) of the starved ultramicro-cell. Starvation of *Vibrio* sp. S14 cells was shown to promote protein synthesis-dependent resistance against autolytic cell-wall degradation, and the proteins involved in autolysis resistance would make the starved cells resistant to the deleterious effects of the activity of autolytic enzymes during non-growth (Nystrom & Kjelleberg, 1989), a feature which must be overcome during the transition to a reproductive state. The function of some maturation-specific proteins may be to degrade specific starvation-inducible proteins involved in the

generation of a non-replicative nucleoid (e.g. DNA-binding proteins) and in the mechanisms of autolysis resistance. Such specific proteases which may be directly involved in autolysin activation (Mencer & Blankenship, 1971) were suggested to be involved in the initial events in the germination of *Bacillus cereus* spores (Boschwitz *et al.*, 1985).

The results obtained here showed that several stresses imposed on the cells, such as heat shock, ethanol and oxidative shock, cadmium chloride treatment and osmotic shock induced significant changes in the protein fingerprints of *A. hydrophila* whole cells, periplasmic proteins and membranes on two-dimensional gel electrophoresis separations. Several proteins were found to be newly synthesized when *A. hydrophila* cells were subject to these stress conditions in lake water microcosms. It suggests that the synthesis of stress-inducible proteins appears to develop necessary responses capable of enhancing the resistance of the cells during exposure to the stresses, and thus the molecular changes that *A. hydrophila* cells undergo seems to make them more hardy with respect to these stresses. Although heat shock, ethanol and oxidative shock, cadmium chloride treatment and osmotic shock each produced its own individual pattern of protein induction, the overlap among stress proteins induced by different agents in *A. hydrophila* was extensive. Several proteins were commonly induced between two or three stresses, which indicates that common proteins induced by many different forms of stress probably produce a resistant effect by stabilizing and protecting the cells against the general stress given to *A. hydrophila*, while individual stress-inducible proteins seem to specific to the individual stress that is given to the cells.

In this study, the fact that each stress induces a characteristic group of proteins along with a smaller number of proteins that overlap with other stress proteins might be explained by the work of Morgan *et al.* (1986), who investigated the significance of the overlaps among the different stress responses and suggested that the overlaps do not result from the generation of a common

intracellular signal, but rather that the genes coding for the proteins that can be induced by several stresses contain the cis-acting regulatory sequences required for several forms of regulation. Spector *et al.* (1986) revealed a considerable overlap among proteins induced under the various starvation conditions but only minimal overlap between the starvation proteins and heat shock or anaerobic proteins. This suggests that major differences in the regulatory circuits controlling these stimulons. However some interconnecting regulation must exist for specific proteins since some responded to both a starvation condition and heat shock or anaerobiosis.

The data in this work further showed that several proteins were produced time dependently under stress conditions examined such as heat shock for whole cells and ethanol shock for periplasmic proteins, and overproduced, as shown by densitometric measurements for certain proteins, in the presence of stresses such as heat shock for whole cells and heat shock, ethanol shock, oxidative stress and cadmium adaptation for periplasmic proteins. This probably suggests that a function of sequential induction and overproduction of proteins is to generate the protective effect for *A. hydrophila* by reaction of the time dependent protein synthesis and by gradual intensification of the synthesis of necessary proteins against the lethal effects of certain environmental stresses such as those tested here. In particular, a large intensification of several periplasmic proteins of *A. hydrophila* was generated by heat shock and ethanol shock, and a sequential increase in intensity of some periplasmic protein fingerprints was also produced during 1 hour exposure to ethanol stress, indicating that these proteins intensified appeared to play an essential role for the survival of *A. hydrophila* during heat and ethanol stress.

The effect of heat-shock inducers on homoserine transsuccinylase was examined by Ron *et al.* (1990), who showed that the cellular level of homoserine transsuccinylase increases substantially when heat shock response was induced by a temperature shift or by the addition of ethanol, and in addition that this enzyme

plays a role in controlling the maintenance of balanced growth rate at elevated temperatures. Spontaneous DNA damage due to heat or oxidative processes appears to be a serious problem with which organisms must cope. Both heat and oxidative stress cause base alterations which result in apurinic and apyrimidinic sites. A function of the heat-shock response, and/or thermotolerance, may be to protect against the lethal effects of certain environmental agents known to cause DNA damage. Maintenance of the replicative integrity of DNA at high temperatures would allow a cell to survive heat stress more efficiently than a cell without such a process, and might result in the high degree of conservation seen in the heat shock response (Delaney, 1990). Farr and Kogoma (1991) have reported that oxidative stress induces some of the proteins that are also induced under other stress conditions, such as heat shock and DNA damage. In particular, heat shock proteins, GroES and GroEL have been shown to be induced by both peroxide and superoxide mediated oxidative stresses as well as heat shock, starvation, SOS at least in *E.coli*. Another heat shock protein, DnaK, has been shown to be induced by treatment with hydrogen peroxide, nalidixic acid, UV irradiation and starvation. Further an SOS protein, RecA, can be induced by both types of oxidative stress but not by heat shock. These observations clearly indicate that several stress responses overlap and that the extent of the overlap varies in different overlapping responses.

Jouper-Jann *et al.* (1992), who studied prestarvation-induced cross protection in marine *Vibrio* sp. and in *E. coli*, suggested that starvation-induced cross protection against heat stress was detected not only for the initial 24 h of starvation, but also during the entire period of nine days of starvation. While short-term starvation gives a very significant degree of protection, prolonged periods of non-growth in fact offer increased protection although after different time periods for the different strains and, for *E.coli* strains depending on the salinity of the starvation regime. They suggested that both *de novo* protein synthesis dependent-starvation and , with the time of starvation, *de novo* protein

synthesis independent-starvation induced protection against heat stress in these strains.

Starvation-induced cross protection against heat or oxidative challenge in *E. coli* K12 was examined by Jenkins *et al.* (1988). Glucose-starved or nitrogen-starved cultures of *E. coli* exhibited enhanced resistance to heat or oxidative challenge, compared with their exponentially growing counterparts. The degree of resistance increased with the time for which the cells were starved prior to the challenge, with 4 h of starvation providing the maximal protection. Protein synthesis during starvation was essential for these cross protections, since chloramphenicol addition at the onset of starvation prevented the development of thermal or oxidative resistance. Starved cultures also demonstrated stronger thermal and oxidative resistance than did growing cultures adapted to heat, hydrogen peroxide, or ethanol prior to the heat or oxidative challenge. Two-dimensional gel electrophoresis revealed that subsets of the 30 glucose starvation proteins were also synthesized during heat or oxidative adaption, including three proteins common to all three stresses. Most of the common proteins were among the previously identified Pex proteins (Schultz *et al.*, 1988). Induction of starvation proteins dependent on cAMP was not important in these cross protections, since an *E. coli* mutant AMS 2 (Δ cya-854), which is unable to synthesize cAMP, exhibited the same degree of resistance to heat or oxidative stress as the wild type parent did during both growth and starvation.

Clark and Parker (1984) carried out two-dimensional gel electrophoretic analysis of the effect of the osmolarity of the growth medium on the protein composition of *E. coli*. They found that only three proteins were synthesized at preferentially higher rates in response to hyperosmotic shock. None were synthesized at detectably increased rates when the cells were exposed to hyposmotic shock. There are only few genes whose transcription is osmotically regulated. Botsford (1990) proposed, however, that at least 41 proteins were found in greater amounts when cells grow with osmotic stress, and

31 major proteins appeared to decrease under osmotic stress, indicating that the response of the bacterium to osmotic stress involves induction of some proteins and repression of others. 61 % of the proteins that appear to be stimulated by salt stress were found in *E.coli* strains except for the *cya* (adenylate cyclase) allele indicating there is no obligatory requirement for cAMP. Jenkins *et al.* (1990) examined the cross-protective effect of starvation against osmotic stress in *E.coli* K12. Starved cultures showed higher osmotic resistance than did similar cultures challenged during logarithmic growth and starvation proteins important in osmotic resistance were found to be cAMP independent for their induction during starvation. The cross protection that starvation conferred against osmotic challenge depended on *de novo* protein synthesis during starvation, since the addition of chloramphenicol at the onset of starvation prevented the development of osmotic protection.

Finally the results show the exact location of alkaline phosphatase of *A.hydrophila* on two-dimensional electrophoresis separations. Alkaline phosphatase, which was identified by colorimetric method using α -naphthyl- PO_4 , was shown to have a molecular weight of about 55,000 with isoelectric pH of about 4.6. No papers have so far tried to identify alkaline phosphatase of *A.hydrophila* by the use of colorimetric reagent, α -naphthyl- PO_4 , which turned out to be a good method for identification of alkaline phosphatase. However, to obtain the understanding on this enzyme in more detail, further investigations such as western blotting and N-terminal sequencing analysis are considered.

Chapter 8

General discussion

Aeromonas hydrophila has been known for a long time to be an opportunistic pathogen for humans, causing wound infections and sepsis. Enterotoxigenic properties have been revealed in representative isolates of *A. hydrophila* and the observation that gastroenteritis could be caused by *Aeromonas* species has resulted in the recognition of these organisms as potential enteric pathogens. *A. hydrophila* is ubiquitous in river and fresh water lakes and frequently observed in both chlorinated and nonchlorinated water supplies. A number of cases of diarrhoea caused by *Aeromonas* species has led to the suggestion that the disease-causing organisms originated in the water supply (Schubert, 1967; Gracey *et al.*, 1982; LeChevallier *et al.*, 1982; Burke *et al.*, 1984a,b; Van der Kooij & Hijnen, 1988). In aquatic ecosystems, the individual and combined effects of a number of environmental factors have been suggested to influence the survival of microorganisms. These include temperature (Barcina *et al.*, 1986), predation (Enzinger & Cooper, 1976), solar radiation (Barcina *et al.*, 1990), nutrient competition (Flint, 1987), nutrient deficiency (Van der Kooij & Hijnen, 1988) and other factors such as sedimentation, salinity, heavy metal concentration, dissolved oxygen concentration, and turbidity.

In this study the survival of *A. hydrophila* in lake water microcosms was studied. These microcosms were amended with a variety of nutrients and other chemicals (which could cause stress) in order to investigate the interrelationship of the starvation and stress-related responses. There was a small difference between the survival times of *Aeromonas* in filtered and non-filtered water samples under starvation conditions and in samples amended with cycloheximide suggesting that protozoa could have a small effect on the survival of *A. hydrophila* in untreated water. Further sterilization of lake water by autoclaving led to a greater increase in the survival time than that observed in filtered samples. This suggests that the existence of natural microflora plays a significant role in the survival of *A. hydrophila* in lake water, and that protozoan predation is one of the reasons for the disappearance of *A. hydrophila* in this microcosms. It has been

reported that ultramicrobacteria and bacteriophage which are able to pass through 0.45 μm membrane filter probably affect the survival of *E.coli* in river water (Flint, 1987). The importance of nutrient competition with natural microflora on bacterial survival in fresh water was also suggested by the work of Flint (1987) and Scheuerman *et al.*(1988). In the study to investigate the effect of nutrient amendments, the addition of nutrient sources to lake water microcosms, for the most part, had a positive effect on *A. hydrophila* survival, indicating the importance of nutrients on bacterial survival and growth in aquatic systems. The addition of synthetic sewage to filtered-autoclaved lake water led to growth even at very low concentrations. This indicates the capability of the cell to utilize the organic component of synthetic sewage, even at 15°C.

The addition of carbon sources at 5 to 500mg-C/l to untreated water led to an increase in survival time and also allowed the population to increase. In filtered-autoclaved water, carbon amendments had a more pronounced effect on cell growth. This suggests that *A. hydrophila* cells are carbon limited in these particular lake water samples. The cells could compete for these carbon substrates with other microorganisms naturally resident in the lake water then they shall be able to survive in the natural environment. To compete successfully organisms need an efficient uptake system, i.e., relatively low K_s value. *A. hydrophila* has a lower K_s than many *Pseudomonas* species for glucose (Van der Kooij and Hijnen, 1988). With phosphate amendments *A. hydrophila* survival and growth increased in proportion to the concentration of phosphate in both untreated and filtered-autoclaved lake water, suggesting that *A. hydrophila* has an efficient uptake system for phosphate in a phosphate-limited aquatic environment. The addition of nitrogen sources, particularly casein, ammonium sulphate, and amino acids, had a large effect on *A. hydrophila* survival in untreated, Whatman-filtered and filtered-autoclaved lake water. The longer survival of *E.coli* in sewage polluted-river water was due to the presence of nitrogen compounds (Flint, 1987). *A. hydrophila* appear to have an effective

affinity system for amino acids, which allows them to compete successfully for these substrates and results in longer survival. The importance of ammonium ion or amino acids to the survival of bacteria in aquatic environments has been shown previously by a number of investigators (Kirchman & Hodson, 1984; Amako *et al.*, 1987; Lim & Flint, 1989).

The effect of substances released from microorganisms on *A. hydrophila* survival was examined. Organic compounds excreted from *Flavobacterium* and the cyanobacterium, *Anacystis*, enhanced survival in addition to cell growth in numbers, both incubated in the dark and under fluorescent light. Organic products from algae were also found to produce a favourable effect on the survival. It is likely that these excreted compounds are organic substrates, such as glycogen, polysaccharide, amino acids and dissolved organic carbon, and act to enhance cell viability and growth, or as light-absorbing materials, like humic and fulvic acids to relieve cell damage from bactericidal effects induced by fluorescent light (Feuillade *et al.*, 1986; Davies & Evison, 1991).

The physiological and morphological changes in *A. hydrophila* under different stress conditions were examined. When the organisms were exposed to starvation conditions, the viable counts were temperature dependently decreased with the most rapid decline at 37°C. However total counts measured by epifluorescence microscopy at this temperature showed no changes in cell numbers from the initial cell population during starvation. This fact indicates that the difference between viable count and total count occurred by cell death but not lysis or by the cells entering a viable but nonculturable state. Further study examining the respiring ability of the cells proved clearly the entry of *A. hydrophila* into viable but nonculturable phase under starvation stress by the fact that at 37°C, viable counts dropped to less than 1% within 2 d but more than 60% of the cells still showed respiration ability. Other stresses, such as osmotic and cadmium shock together with nutrient deprivation yielded essentially similar data. Although the cells lost their ability to grow on agar medium under

osmotic stress, they could recover their viability and respiring ability by the elimination of sodium chloride or by the addition of osmoprotectants, such as betaine or proline. The morphological reduction in cell size occurred temperature dependently under starvation stress and nutrient amendments had a protective effect on the reduction of cell size. These results indicate that *A. hydrophila* protect themselves by the changes in physiological and morphological state of the cells during exposure to unfavourable stress conditions in aquatic environments.

Alkaline phosphatase has been regarded as an enzyme important to the survival of bacteria when the cells are subjected to the lack of inorganic phosphate in the growth medium (a form of starvation stress). It was therefore expected that the enzyme would be produced when bacteria were grown under starvation conditions. Alkaline phosphatase activity increased over a 36 d starvation period at temperatures between 4° and 37°C in cells grown under phosphate limitation. This increase actually occurred without an increase in cell numbers and at 4°C, a temperature at which protein synthesis would not be expected to occur. The increase in activity was also seen at 37°C although the viable count reduced to almost undetectable levels whilst activity was increasing. This increase could be due to starvation induced changes in the outer membrane of the cells allowing the substrate easier access to the enzyme located in the periplasmic space or be due to viable but nonculturable cells which are still capable of producing alkaline phosphatase under starvation conditions but are unable to grow on agar plates, particularly cells incubated at 37°C.

The addition of nutrients, such as single carbon sources, casein and some amino acids, to filtered-autoclaved lake water microcosms increased alkaline and acid phosphatase activities in cells grown in a phosphate limited medium (PLM) and in a phosphate rich medium (PRM), suggesting the derepression of enzyme activity is probably connected to the ability of the cells to carry out protein synthesis and the maintenance of this essential metabolic activity is enhanced

under starvation conditions by the addition of selected nutrients. However some nitrogen sources, such as ammonium sulphate and sodium nitrate, did not cause an increase in alkaline and acid phosphatase activity in PLM and PRM cells although *A. hydrophila* survival was enhanced. This also indicates that the effect of these substrates is not on cell metabolism and rather seems to stabilize the cells to prevent loss of viability. When PLM cells were subjected to osmotic stress, the alkaline phosphatase activity increased as salt concentration increased. No increase in phosphatase activity occurred in PRM cells, indicating that osmolarity did not cause derepression of alkaline phosphatase synthesis but led to further synthesis of the enzyme if this was already derepressed. Alternatively the increase could be due to changes in outer membrane permeability under osmotic shock, which resulted in easier access for the substrate to the enzyme in the periplasm.

The addition of betaine to PLM and PRM cells increased the activity of both alkaline and acid phosphatase under osmotic shock. This indicates that betaine, which is an amino acid analogue, appears to exert a similar function as some amino acids did to stimulate the further production of both alkaline and acid phosphatases. Alkaline phosphatase formation in PLM cells was strongly inhibited by some oxyanions, such as phosphate and vanadate. Almost complete repression of alkaline phosphatase activity occurred with phosphate amendment, whereas acid phosphatase activity was highly increased with phosphate addition.

It has been known that extracellular protease played an important role in supplying the cells with available amino acids, and bacterial survival and growth is closely associated with protease production in the aquatic environment. Here extracellular protease activity was measured to examine the effect of starvation stress, nutrient amendments, nutrient deficiency and osmotic shock on *A. hydrophila* survival. Extracellular protease production was increased in proportion to the temperature during a 35 d starvation period. Although the viable counts at 37°C rapidly declined to less than 1% of the initial cell density

during the starvation period, the exoprotease concentration remained actively constant. This suggests that when *A. hydrophila* are stressed by the exposure to nutrient limited environments, the production of extracellular protease, known to be one of the scavenging enzymes for the digestion of substrates, seems to be increased. The purpose of the enzyme production is probably to degrade the potential polymeric nutrients residing in the surrounding area to provide readily utilizable substrates for bacterial survival and growth. The addition of nutrients to starved lake water enhanced extracellular protease activity. Particularly lactose and casein amendments induced a large increase in extracellular protease activity with an increase in survival times. This suggests that the production of protease aids bacterial survival by releasing utilizable carbon and nitrogen sources in lake water. It was suggested that the difference in protease production with different nitrogen sources may reflect differences in amino acids able to induce the protease (O'Reilly & Day, 1983), and may occur by variations in the quantity of specific inducer(s) (McKellar, 1982). Betaine addition to lake water amended with salt also had a large effect on the production of extracellular protease, suggesting that betaine, as an amino acid analogue, seems to have a function as an inducer for enzyme production.

The effect of starvation stress, nutrients and cell density in lake water on plasmid transfer was also studied. The transfer of plasmid was shown by an increase in the numbers of transconjugants under starvation stress. However no detectable number of transconjugants were obtained at 15°C or below. This suggests that conjugal transfer is able to occur under starvation condition but the transfer by conjugation would be a rare event *in situ* where the lake water temperature would be expected rarely to exceed 15°C. It is likely that low temperature exerts a negative effect on mating-pair formation between donors and recipients. The addition of nutrient sources to lake water led to an increase in the number of transconjugants, suggesting that plasmid transfer by conjugation is greatly affected by the availability of organic substrates in the mating medium.

It was further shown that the deficiency of nutrients is probably a major reason for reduced plasmid transfer in lake water (Jones *et al.*, 1991). Transconjugant numbers recovered were dependent upon initial concentration of donor and parent cells in lake water.

Finally two-dimensional gel electrophoresis separations were used to examine the changes in protein fingerprints of whole cell extracts or of the periplasm of *A. hydrophila* under starvation and other stress conditions. Starvation stress led to an induction of several starvation specific proteins and a repression of some proteins at all temperatures from 4° to 37°C up to 28 days. 30 proteins were commonly synthesized at temperatures from 4°C to 25°C and 13, 2 and 7 proteins were synthesized temperature specifically at 4°, 15°, and 25° C respectively during 4 h starvation. The new synthesis or disappearance of proteins during the starvation period shows that the cells rearrange their intracellular proteins to enable further growth and survival to occur in response to starvation stress. These data also showed that some proteins were sequentially and transiently induced or repressed, and intensified time dependently, indicating that those changes in protein patterns are likely to be an essential process of adaptation of *A. hydrophila* for survival in response to starvation stress. The synthesis of new proteins in the absence of nutrients and energy sources seems to be involved in the capture and transport of substrates resident in the surrounding environment.

Nutrient additions to lake water caused a sequential synthesis of new proteins, suggesting that these proteins differ from starvation inducible proteins and are produced by nutrient upshift. The imposition of other stresses, such as heat, ethanol, cadmium, sodium chloride, and hydrogen peroxide to *A. hydrophila* brought significant changes in protein fingerprints. Five major proteins in whole cell extracts were gradually intensified during 1 hour under heat shock. It suggests that the process of synthesis and identification of stress inducible proteins probably correlates with molecular changes for development of the

necessary response able to enhance *A. hydrophila* resistance during exposure to the stresses. The N-terminal sequences of two proteins, which were significantly intensified under ethanol and heat stress conditions were also elucidated. Alkaline phosphatase was identified with a molecular weight of about 55 kDa and an isoelectric point of about pH 4.6 on 2-D electrophoresis separations.

Overall this project has been performed to investigate the physiological and morphological changes concerned with *A. hydrophila* survival under starvation and other stresses. The other stresses were used to examine the comparative effects on the bacterial cells. On the basis of the result of this study, the following is concluded that under starvation stresses, *A. hydrophila* (i) entered a viable but nonculturable phase with a reduction in cell size, (ii) induced the production of a series of scavenging enzymes, such as phosphatase and exoprotease, (iii) was effective in genetic transfer, and (iv) suffered from a time dependent changes in protein composition. 2-D separations allowed the identification of the exact location of alkaline phosphatase which is considered as an important enzyme for *A. hydrophila* survival under starvation conditions. Further work would allow a more detailed investigations of other starvation-specific and stress-inducible proteins by techniques such as western blot and N-terminal sequencing analysis.

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